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# CONTENTS

## No. 1 JANUARY

E. J. LUND. The relations of Bursaria to food. I. Selection in feeding and in extrusion. Eight figures.....	1
ALICE M. BORING AND RAYMOND PEARL. The odd chromosome in the spermatogenesis of the domestic chicken. Six plates.....	53
CHARLES PACKARD. The effect of radium radiations on the fertilization of Nereis.....	85
JOHN C. PHILLIPS. A further study of size inheritance in ducks, with observations on the sex ratio of hybrid birds. Seven charts.....	131
GEORGE L. STREETER. Experimental evidence concerning the determination of posture of the membranous labyrinth in amphibian embryos. Thirty-six figures.....	149

## No. 2 FEBRUARY

E. C. MACDOWELL. Multiple factors in Mendelian inheritance.....	177
HENRY LAURENS. The reactions of normal and eyeless amphibian larvae to light. Two figures.....	195
GEORGE ALFRED BAITSELL. Experiments on the reproduction of the hypotrichous Infusoria. II. A study of the so-called life cycle in Oxyticha lallax and Pleurotricha lanceolata. Sixteen figures (one plate).....	211
LORANDE LOSS WOODRUFF. On so-called conjugating and non-conjugating races of Paramecium. One figure.....	237
ARTHUR WILLIAM MEYER. The supposed experimental production of hemolymph nodes and accessory spleens. V. Studies on hemal nodes.....	241

## No. 3 APRIL

N. E. McINDOO. The olfactory sense of the honey bee. Twenty-four figures	285
CLAUDE W. MITCHELL AND J. H. POWERS. Transmission through the resting egg of experimentally induced characters in <i>Asplanchna amphora</i> . . . . .	347
W. C. ALLEE. Certain relations between rheotaxis and resistance to potassium cyanide in <i>Isopoda</i> . . . . .	397
C. M. CHILD. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. VII. The stimulation of pieces by section in <i>Planaria dorotocephala</i> . Four figures . . . . .	413
G. H. PARKER. On the strength and the volume of the water currents produced by sponges . . . . .	443

## No. 4 MAY

H. H. NEWMAN. Modes of inheritance in teleost hybrids. Thirty-eight figures	447
MARGARET MORRIS. The behavior of the chromatin in hybrids between <i>Fun- dulus</i> and <i>Ctenolabrus</i> . Thirty-six figures (five plates) . . . . .	501
FRANK R. LILLIE. Studies of fertilization. VI. The mechanism of fertili- zation in <i>Arbacia</i> . One figure . . . . .	523
RALPH S. LILLIE. Antagonism between salts and anesthetics. IV. In- activation of hypertonic sea-water by anesthetics . . . . .	591
JONATHAN RISSE. Olfactory reactions in amphibians. One figure . . . . .	617

# THE RELATIONS OF BURSARIA TO FOOD

## I. SELECTION IN FEEDING AND IN EXTRUSION

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EIGHT FIGURES

### CONTENTS

Introduction.....	2
Action of the structural mechanism for feeding and the selection of food...	4
1. Rejection.....	5
2. Acceptance.....	5
Formation of the vacuole and elimination of residues.....	7
Measurement of the amount of food eaten, and method of experimentation...	8
Internal relations affecting the feeding process.....	10
1. The relation of the physiological state of the organism to the feeding process.....	10
2. Changes in the physiological state as shown by using the feeding process as an index.....	16
3. Other causes of individual variation.....	19
The external relations of the feeding process.....	20
1. Effects of external factors on feeding.....	20
a. Concentration of the food supply.....	20
b. Effect of mechanical stimulation and mechanical injury on feeding.....	21
c. Temperature.....	23
d. HCl and NaOH.....	24
e. White light.....	24
f. The electric current.....	26
Selection of food and the factors concerned.....	29
1. Experiments with stained and unstained yolk.....	30
a. Saffranin.....	30
b. Janus green.....	34
c. Hematoxylin.....	37
d. Congo red.....	39
e. Sudan III.....	40
f. Stale yolk.....	40
2. The basis for and the nature of the selection of food in Bursaria.....	41
The relation of Bursaria to digestible and non-digestible substances.....	43
1. External relations.....	43
2. Internal relations.....	44
Summary.....	51
Literature cited.....	52



## INTRODUCTION

While studying the phenomena of regeneration and structural regulation in *Bursaria*, it became desirable to know something of the relations of this organism to its food and of the processes which the solid food material undergoes in its passage into and through the cell and in the elimination of residues. This paper aims to present the first part of these observations and to make a general survey of the relations of this organism to food, leading up to a more detailed study of these and certain other problems of the cell as found in this unicellular animal.

*Bursaria* was found to be much more favorable for the investigation of these phenomena than the smaller infusoria such as *Paramecium*. No study has heretofore been made of the relations of *Bursaria* to food, so that the facts herein presented are new. The present investigation attempts not only to determine qualitatively whether the relations to food are similar to those more or less known for *Paramecium*, *Stentor*, *Vorticella* and other infusoria, but more particularly to work out and express these relations in a more quantitative way than has been done heretofore. It was found that certain kinds of experimental tests such as those on the rate of digestion, could be made upon this form, which it would have been difficult or impossible to carry out on smaller unicellular organisms. Its very large size offers a singular opportunity for easy manipulation in many kinds of work. When in a clear medium it is readily visible to the naked eye at a distance of six or eight feet and individuals may be transferred singly with a pipette without the aid of any magnifying instruments.

*Bursaria* occurs not infrequently in cultures brought from ponds in the vicinity of Baltimore, though it is less common than many other Protozoa. It can readily be cultivated in large culture dishes in the laboratory. In this way I have had abundant material at my disposal for many months. The method of cultivation has been simply the inoculation of an infusion of timothy hay in tap water from the wild culture; by several inoculations at different times one usually succeeds in obtaining large numbers. Since the food of this organism is not bacteria,

so far as I have yet observed, but a variety of other ciliates, flagellates and rhizopods, it is difficult to find a culture medium which can be readily manipulated, and hence, pure line cultures can not be obtained so readily as of a form like *Paramecium*. The problem of pure line cultivation of this organism will not be dealt with in this paper. The material for use in the following experiments has all been obtained from mixed or 'wild' cultures, though the reinoculations from the single parent culture brought into the laboratory seven months ago resulted in a small number of pure lines living side by side in the cultures.

It is, in fact, preferable in some ways to use material from such wild cultures for the kind of experiments to be considered in this paper.

Even without the aid of pure cultures or the application of statistical methods to wild cultures it soon became apparent that there are actually at least two very distinct races of *Bursaria* which differ in several diverse characters, physiological as well as morphological.<sup>1</sup> One form, which under certain food conditions has a tail, has been used exclusively in these experiments, since the other form, collected at the same time and lacking a tail, died out early in the experiments.

I have observed the following organisms to be eaten and digested by *Bursaria*: *Chilomonas*, *Colpidium colpoda*, *Vorticella* and some of its relatives, *Oxytricha*, *Stylonychia*, *Arcella*, *Stentor*, *Paramecium*, *Stephanodiscus*, and some kinds of rotifers. Only once have I observed bacteria to be eaten, and that time in the form of zoogloea. It is, however, certain that bacteria form only a small part, if any, of the usual diet of this organism. The smaller ciliates, flagellates and rhizopods are the favorite article of food. The larger organisms, such as *Stentor*, are seldom successfully captured. *Paramecium* is quite commonly eaten, though *Bursaria* does not seem to thrive well on this food. Occasionally rotifers are eaten and it was observed on several occasions that these may remain alive within the vacuole

<sup>1</sup> I have been unable to find reference in the literature to more than one form of *Bursaria*. A consideration of the problems connected with the existence of diverse 'races' of *Bursaria* will be left for a later time.

for as long as five hours before they are killed. It is, however, plainly evident when one follows the development of wild cultures from day to day that some forms are eaten in greater numbers than others and if the smaller forms, such as *Colpidium*, *Vorticella* and *Arcella*, are present in abundance along with such forms as *Paramecium*, *Stentor* and *Stylonychia*, the former kinds serve exclusively as food for *Bursaria* while the latter are rarely eaten. When the cycle of development of the culture comes to the stage where, for example, *Paramecium* is in superabundance, then the body of *Bursaria* may be more or less filled with *Paramecia*. In contrast to the above mentioned forms, *Spirostomum ambiguum* was always rejected. It was often seen to be taken into the oral pouch but invariably was thrown out again, while *Paramecia* present in the same culture were readily eaten by the same *Bursaria* individuals at the time of the observations. This is the only case where *Bursaria* was seen definitely to discriminate between two different forms of Protozoa.

By simple methods of observation like the above, it would be impossible to determine just what the principle and the factors are that determine whether *Bursaria* will feed on only one or several or all of these forms if they be present in all the cultures simultaneously, which of course they often are. It is with the object of elucidating these and certain related questions that the following comparatively simple experiments have been performed, by limiting and determining to a high degree the conditions under which this organism will react to food.

#### ACTION OF THE STRUCTURAL MECHANISM FOR FEEDING AND THE SELECTION OF FOODS

An account of the food relations of *Bursaria* requires us to examine in some detail the objective processes involved in feeding; these are very striking. The highly developed oral apparatus with its large cilia, when in operation during the feeding process, may easily be observed. When the organisms are fed on such substances as yolk or starch they usually sooner or later become quiet for a time, and settle to the bottom of the dish or stick to the surface film of the water, then they may be

observed under a high power of a binocular. Granular substances of different chemical or physical properties may be placed in the medium and the path of each individual particle may be easily observed. Mixtures of these substances may also be made and the paths of the different kinds of particles may be determined.

The different paths of particles which come into varying relations with the organism are shown by the arrows in the outline drawings of figure 1. The paths of the arrows are correct representations of the paths taken by the different kinds of particles. In general the paths taken by particles may be distinguished according to the following outline:

*1. Paths of rejection*

- a. Path of total rejection, arrows A
- b. Path of rejection of larger particles taken into the oral apparatus, arrows B
- c. Paths of rejection of smaller particles taken into the oral apparatus, arrows  $C_1$  and  $C_2$ .

These paths may also be slightly modified by a combination of the avoiding reaction with the different rejection reactions.

*2. Path of acceptance of large and small particles (large arrows D)*

Path A is taken by those particles which under conditions hereafter to be considered (p. 29) never enter the oral apparatus and are only drawn towards the body by the current; for example, very toxic particles of yolk. Path B is always taken by those particles which are too large to pass out by way of path  $C_1$  and  $C_2$  and must be passed back to the exterior by the same way as they were taken in, in order for the organism to get rid of them at all. This may be illustrated by the larger properly treated grains of hard boiled yolk. The path represented by the arrows  $C_1$  and  $C_2$  has considerable range of variation in part of its course. It may be illustrated by cornstarch grains; these are of convenient size. The variations in the course of these particles may be divided roughly into two main divisions; some follow the dotted arrows  $C_2$  and never directly retrace any

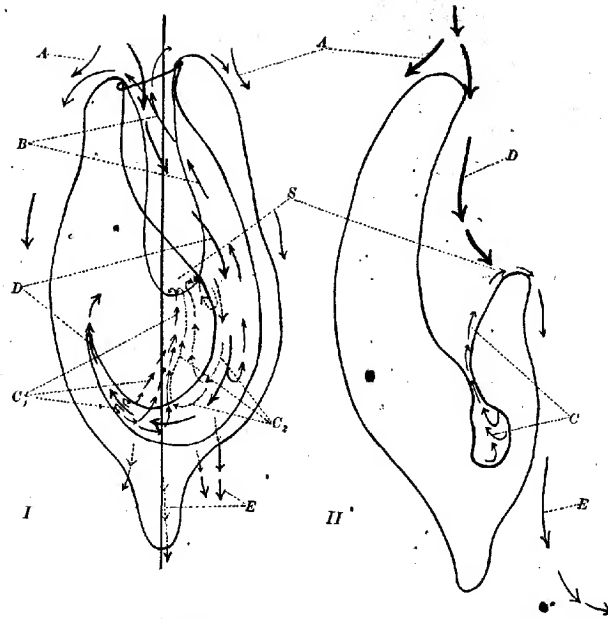


Fig. 1 I, Outline drawing from dorsal side of Bursaria, to show position of the oral pouch in the body, and the paths of variously rejected and accepted particles. A, path of total rejection; B, path of rejection of large particles which are too large to pass out by way of the oral sinus, S. C<sub>1</sub> and C<sub>2</sub>, paths of rejection of small particles; these pass out by way of the oral sinus, S; D, path of acceptance. II, Outline drawing of sagittal section, in the plane through the body represented by the straight line through I. A, path of total rejection; D, path of entrance of all particles taken into the oral pouch, same as first part of path D, I. C, path of rejection of small particles, same as C<sub>1</sub> and C<sub>2</sub> of I. E, I the same as E II, direction of rejected particles, C, I, and C<sub>1</sub>, C<sub>2</sub>, II.

part of their former path; some pass up into the proximal end of the oral pouch but are rejected and returned to the outside by way of the continuous arrows C<sub>1</sub>. All the paths of rejection under C<sub>1</sub> and C<sub>2</sub> converge and lead to the exterior by way of the oral sinus, figure 1, I and II S.; they then pass backward under the posterior ventral side, as shown by arrows E. There is but one path of acceptance for both large and small particles.

Figure 1, II, is a sagittal section through the body in the plane indicated by the straight line through figure 1, I; it shows the path of total rejection, arrows A, the path of entrance (by heavy arrows D) and the path of rejection of smaller particles (by arrows C).

At the point of entrance into the endoplasm the transport of the accepted particles is brought about not alone by the cilia but also if not exclusively, in the case of larger particles, by a peristaltic wave in the wall of the oral cavity behind the particle pushing it into the body.

#### FORMATION OF THE VACUOLE AND THE ELIMINATION OF RESIDUES

The vacuoles when formed always contain some liquid, though at times the amount may be very small. The size and shape of the vacuoles varies greatly and depends upon the kind of food eaten, and upon many other conditions, as will be shown. Often the food forms large irregular masses, which in the case of fresh yolk may so completely fill the body that after a half-hour or more of feeding the dorsal side of the body cortex is burst open and the food mass is extruded. The opening then closes and the organism again assumes its usual shape. The rate of formation of the vacuoles is intimately bound up with the same complex conditions which determine their size and shape. The circulation of the vacuoles in Bursaria is not reducible to any definite order, such as has been shown to exist more or less definitely in *Paramecium*, by Metalnikow ('12) and others, and in *Carchesium* by Greenwood ('94). The vacuoles often become lodged in one place and there digestion is completed. This may often be seen in cases where fat-extracted yolk particles become lodged in the tail. During digestion and resorption the large vacuoles usually become smaller and any residual contents are finally extruded. The residues are always extruded from a small area on the mid-dorsal side of the body of the organism. This may readily be demonstrated by feeding the animals chinese ink. The changes which take place in the food vacuole from its formation to its disappearance will be considered in detail in a later paper.

MEASUREMENT OF THE AMOUNT OF FOOD EATEN AND METHOD  
OF EXPERIMENTATION

In order to express quantitatively the relations of *Bursaria* to food, it is necessary to obtain a reliable method for measuring the amount of food taken in a given length of time, under given conditions. The unit of volume employed was that of one grain of fresh hard-boiled yolk of hen's egg. The eggs were boiled fifteen minutes. These grains are readily eaten by *Bursaria* and may be obtained of an approximately uniform size. It is necessary to deal with suspensions of such grains, having a uniform concentration (that is, containing the same number of grains to a given volume). For this purpose stock suspensions were made up on successive days from yolk of the same egg: these were made uniform by making them up in vials of the same size and comparing each with a standard concentration kept in a vial of the same size. Various known grades of concentration were then made up by adding a known volume of the stock suspension to 5 cc. of water in a slender dish of 8 cc. capacity. This procedure was found to be sufficiently accurate to avoid the introduction of any observable variation in the amount of yolk eaten in a measured period of time (page 20.<sup>2</sup>) Uniformity in the size of the yolk grains was obtained by repeatedly washing the fresh hard boiled yolk crystals in distilled or tap water and decanting, until the suspension when left to settle leaves a clear supernatant liquid. The smaller grains remain in suspension a little longer than the larger ones and thus may be removed by decantation. Uniformity in size is still further obtained by drawing off the grains from the same level in the clear suspension with a pipette. Some eggs have yolk crystals of more uniform size than others, so that only the eggs best in this respect have been used.

<sup>2</sup> In most of the experiments it was necessary to make up only a single stock suspension, since the animals were fed only once and all the feeding was carried out at the same time. In the case of experiments which required the feeding of yolk on more than one day, however, this standard concentration had likewise to be made up anew each day by comparison with that of the day before.

The uniformity in size of the yolk grains is of course of paramount importance in many of the experiments and for some of the conclusions which will be drawn from them. In order that the degree of uniformity might be tested and indicated quantitatively, a large number of measurements of grains of the prepared yolk suspension were made at different times by means of a stage micrometer. The following shows a typical result of one set of these measurements: 105 grains were measured and the numbers divided at random into three sets of 35 each and the average of the diameters taken. These gave respectively 0.0890, 0.0906, and 0.0837 mm. The range of variation of the diameter was from 0.060 to 0.130 mm. The distribution of the variations are shown by the following figures:

Diameter of grain mm.....	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13
Frequency.....	12	19	16	14	20	13	6	5

By thus being able to obtain a very constant average diameter of a comparatively small number (30 to 50 grains), the errors introduced by the individual variation in size, which in the above example is about as  $\frac{1}{2}$  is to 1, is largely eliminated. In order to remove the objection to experimental results based on the volume of granules of varying size, a large number of individuals (20 to 100, depending upon the purpose of the experiment) were used in each experiment and the number of grains eaten was counted; furthermore the experiments were always repeated whenever there could be any doubt as to the validity or significance of the results. Hence, as will be shown later, the lack of strict individual uniformity of the unit volume is corrected (a) by the fact that the average size of the yolk grain is practically constant, (b) by using a large number of individuals in each experiment, and (c) by repeating the experiment.

Thus having the form, weight and volume of the units of food eaten made practically constant, we may vary one of their properties—as for example, their chemical nature—by letting them adsorb different kinds of toxic and non-toxic substances which are diffusible or non-diffusible in the native medium, tap or distilled water. We may therefore test the responses to variations



in this one property—namely, the chemical nature of the grain—and its effects.

An approximately constant medium was provided by using tap water. This precaution is important, for, as will be shown, the nature of the medium often affects or determines the kind of results which are obtained. Distilled water was also used but it was found that this extra precaution was not necessary in most of the experiments, and since distilled water is toxic if the organisms are left in it too long or the change is too rapid, it could not have been used in many of the experiments, even if it had been otherwise desirable to do so.

The organisms were starved in 400 cc. of tap water for eighteen to twenty-four hours previous to each experiment. At the end of this time they were free from food and residues. Thus an optically clear, active and perfectly normal cell was obtained with which to begin work in all the experiments where uniformity in this respect was desired. All the factors with which we are dealing, except the 'physiological states' of the organisms themselves are known and uniform to within narrow limits, while the one of which we wish to test the effects can be controlled and varied.

#### INTERNAL RELATIONS AFFECTING THE FEEDING PROCESS

##### *1. The relation of the physiological state of the organism to the feeding process*

By the words 'physiological state' is here meant the condition as a whole, of the equilibria in the physical and chemical reaction system, the cell, at a certain time in the duration of its existence.<sup>3</sup>

This condition or state is to be thought of as being limited to the space which the organism occupies; or is, in other words, internal. However, it is obviously absurd for anyone to attempt

<sup>3</sup> This definition is justified because in so far as the facts are at present known, this is the only kind of system with which we have to deal in the cell, and therefore in the present state of knowledge the only logical universal assumption for experimental purposes is to define 'physiological states' in terms of such known systems, until the universality of the assumption is disproved.

a definite and strict separation of the internal and external of any living organism, and especially is this true of the cell. Yet for purposes of presentation, this becomes highly convenient, and it is only for this purpose that the above rough distinction is made here. *When all external conditions are made the same in two experiments which nevertheless give different results, the differences must be attributed to different conditions within the organism,* and it is, as a rule, only in this way that different physiological states are at present practically perceptible.

Differences in physiological state in unicellular animals are made evident most readily in the relations to food, as may be seen from the work of Metalnikow ('12) on *Paramecium* and by Schaeffer ('10) on *Stentor*.

*Bursaria* affords most excellent material for the elucidation of the relation of these dynamic states to the feeding process and of the fact that this relation changes while the external conditions remain constant. These facts are brought out in the following experiments by using both single individuals and large numbers of individuals collectively, at the same time, and analyzing the results.

*The total quantity eaten and the rate of feeding.* Table 1 gives the results of a typical experiment designed to show the difference in the total quantity of food eaten and also the difference in the rate of feeding of *Bursaria* from different cultures.

Material from two different cultures, A and B, was starved twenty-four hours in each of two dishes containing 400 cc. of tap water. 1 cc. of a fresh hard boiled yolk suspension was placed in each of 16 stender dishes of 8 cc. capacity; 5 cc. of tap water was then added to each. Thirty individuals from culture A were placed in each of 8 of the dishes. Alternately with these 8 sets from culture A were placed 8 sets of thirty individuals each from culture B in the other 8 dishes. At the end of the time intervals noted in the table, in each case, the contents (6.5 cc.) of one dish each of A and of B were transferred into a stender dish with 500 cc. of tap water. This stops the feeding. The individuals were then immediately picked out of these large dishes, placed in 8 cc. dishes and killed in Meves' fluid. The

counts of the number of grains contained in each individual were taken at the end of the experiment.

Table 1 shows (1) that *Bursariae* living in the two different cultures differ in the total amount of food eaten in the same length of time. In other cases, of course, individuals from diverse cultures will give identical results so far as feeding is concerned, while two or more different cultures may also differ to a greater extent than the above table shows. Moreover, the amount of food eaten by a given culture may vary at different times. The greater the length of time of feeding (within certain limits) the greater the total amount of food eaten. Not only does the total amount of food taken differ in the two cultures, but what is equally important, (2) the rate of feeding varies with organisms from different cultures. This was observed in numerous other experiments. Under some conditions the animals fill their bodies quickly, while at other times this takes place slowly; or only a small number of grains may be eaten.

The facts are shown most clearly by the curves *A* and *B*, figure 2, representing the number of grains of yolk (ordinates) eaten by the thirty individuals in successive periods of one-half minute (abscissae) throughout the time of the feeding process. Curve *A* is plotted from the results of culture *A* and curve *B* from those of culture *B*, in table 1. The immediate rapid rise of curve *A* shows that the rate of feeding of culture *A* during the first six successive periods of one-half minute each was about from five to twenty times as great as in any of the subsequent fifty-seven minute intervals. A similar high initial rate is also shown by curve *B* (culture *B*), but here the rise to the maximum was not so steep and the rate during the first six half-minute periods was only about from four to ten times the rate during the subsequent fifty-seven half-minute intervals.

In order to show more clearly that the results apply to the individuals taken separately as well as to the averages for all (i. e., to the cultures as a whole) the data may be arranged as in table 2. As this table shows, at the end of sixty minutes all but an insignificant number of animals from each culture had eaten yolk grains: hence, the difference in the amount and

TABLE I  
Showing the results of a comparison of the rate of feeding and the total number of grains of yolk eaten by eight sets of thirty individuals each from two different cultures, A and B

TIME IN MIN- UTES	CUL- TURE	NUMBER OF GRAINS OF FRESH YOLK EATEN BY EACH CELL																				TOTAL NUMBER OF GRAINS EATEN	AVERAGE NUM- BER OF GRAINS EATEN PER INDIVIDUAL	AVERAGE NUMBER OF GRAINS EATEN IN SUCCESSIVE 1/2 HOURS AVERAGE RATE OF FEEDING								
5	A	0	0	0	1	1	2	3	0	1	0	4	0	0	2	2	1	2	1	0	1	0	2	3	0	2	0	3	0	32	1.06	32.0
	B	0	0	1	0	0	0	1	0	0	0	1	2	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	10	0.33	10.0
1	A	1	2	1	0	1	0	4	0	6	1	1	4	6	2	1	0	1	6	2	4	4	8	6	3	0	4	2	0	70	2.33	38.0
	B	0	0	0	1	4	0	1	0	1	2	0	2	0	3	0	0	0	1	0	0	2	0	0	4	0	0	0	25	0.83	15.0	
3	A	10	9	16	20	9	8	3	5	8	6	10	11	2	0	1	5	7	4	0	6	3	1	0	12	0	13	4	7	198	6.60	32.0
	B	0	1	3	1	0	1	0	3	0	2	2	4	0	7	4	0	2	1	3	6	4	3	2	0	4	58	1.93	7.0			
5	A	0	0	10	10	0	9	12	11	0	15	9	12	5	6	4	10	11	7	0	15	10	8	6	7	8	12	11	227	7.56	7.25	
	B	3	3	4	6	1	4	3	2	3	4	0	2	0	1	0	3	0	2	1	0	3	4	0	2	6	2	5	1	71	2.37	3.25
10	A	9	24	10	8	4	9	5	16	6	11	2	3	10	8	4	3	0	3	16	3	14	16	4	7	0	12	9	241	8.03	1.4	
	B	10	7	7	4	4	3	0	4	0	2	3	2	9	2	3	2	0	0	5	1	3	1	2	3	1	6	3	0	91	3.03	2.0
20	A	14	10	15	3	7	10	13	7	4	11	7	0	7	3	2	10	9	15	12	15	5	14	9	8	9	2	0	232	9.73	2.55	
	B	1	4	8	8	1	0	4	3	6	3	0	1	6	1	1	9	9	3	8	0	8	3	4	9	0	0	2	114	3.80	1.15	
30	A	15	16	24	15	0	7	14	5	7	4	16	7	24	3	16	13	15	11	16	0	10	0	9	20	2	4	14	215	10.53	1.2	
	B	6	3	6	0	7	6	2	4	5	2	0	5	4	5	11	0	0	8	4	6	5	9	4	0	6	5	4	117	3.90	0.15	
60	A	16	0	15	8	19	0	8	20	20	5	23	6	12	15	29	9	11	14	6	13	22	13	24	15	16	11	0	19	381	12.70	1.08
	B	6	8	8	14	10	3	8	16	4	6	0	3	0	1	5	4	8	3	8	9	0	6	1	0	12	0	5	0	180	6.00	1.05

rate of food taken by the two cultures was not due to some sporadic difference caused, for example, by a very high rate of feeding by a few individuals and no food eaten by others, but rather to a uniform difference between the sets of individuals from the two cultures. Therefore the results are typical for the individual as well as for the culture as a whole. Moreover, if we calculated the averages of *A* and *B* on the basis of those individuals alone which had one or more grains, the average of *A* would still be greatly in excess of that of *B*.

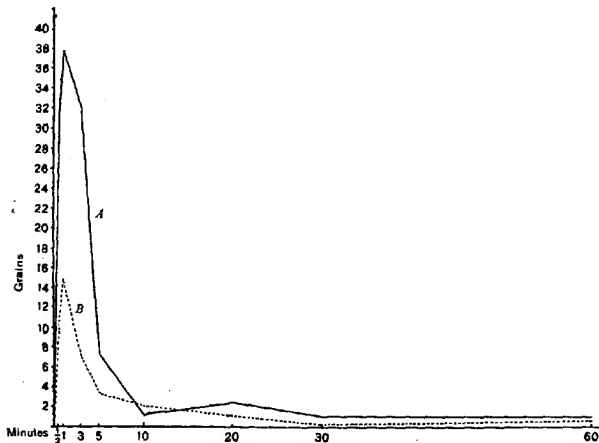


Fig. 2 Showing the rates of feeding by the two cultures *A* and *B*, curves *A* and *B*, respectively. Plotted from the results of table 1.

We may express the variation in the total quantity eaten by the standard deviation of each corresponding group of thirty individuals in *A* and *B*, as is done in the last column of table 2. The reciprocal of the standard deviation ( $\sigma$ ) is a measure of the degree of uniformity among the individuals. It will be noted that there is an increase in the range of variation and the standard deviation with increase in the length of time of feeding; this means that the difference in physiological state among individuals of the same culture finds a fuller and more definite ex-

TABLE 2

*Showing the differences in physiological state of Bursaria from two Cultures, A and B. The feeding process is used as an index*

pression in the results of the experiment as the length of time of feeding is increased. The final total number of grains eaten when the time is long is then a more accurate index of the relation of the physiological state to the feeding process than if the time of feeding is short. The greater this difference in the total quantity of food eaten the greater is the difference in the physiological state of the different individuals. We, therefore, have in the amount of food eaten, if the length of time of feeding is long enough, a fairly good relative measure of the physiological state of the single individual and the differences in the physiological state between different individuals as regards their relation to food at that particular time.

*2. Changes in the physiological state as shown by using the feeding process as an index*

If change in the dynamic conditions of the cœm, as regards the food relation, does occur, this should be observable by a change in the feeding process, and such is indeed the fact. This is shown in table 3. Material from cultures *C* and *D* was starved in tap water for twenty-four hours. Five active individuals were then picked out from each and tested individually. They were fed twenty minutes and each one was observed continuously during the experiment. The number of grains eaten and rejected and the time as called off by the observer were noted.<sup>4</sup> In this way the time record of the relation of acceptance and rejection of food was obtained. The yolk concentration, temperature, and so forth were the same in all the tests.

As the table shows, yolk grains were at first rapidly eaten. At the end of the first few one-half minute intervals the action of the cilia was frequently reversed, thus rejecting the food after it had been taken into the oral apparatus. There was, therefore, a definite change from eating to the rejection of food by the feeding mechanism. This change was more rapid in general, in the individuals from culture *C* than in those from culture *D*.

<sup>4</sup> I am indebted to Mr. K. S. Lashley for kindly aiding me in taking the records of this experiment.

TABLE 3

April 4: CULTURE C

Starved, tap, 24 hours

Tests on individuals

TIME MINUTES	1	1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	SUM	
I { Accepted.				5	1	3	3	3	6	4	5	3	3	6	7	1	1							112
I { Rejected.				3	3	2	3	3	6	7	1	1	7	1	4	3	1	7	2					118
II { Accepted				4	3	2																	11	
II { Rejected				3	2		3	2	6	4	4	1	2	1	4	3	2	3	1	3	2	4	5	2
III { Accepted				3	4	7	3	3															74	
III { Rejected				4	1	2	4	1	2	2	4	3	3	6	1	3	1	3	1				21	
IV { Accepted				3	1	2																	60	
IV { Rejected				3	1	2																	11	
V { Accepted				5	6	1	3																57	
V { Rejected.				1	4	3	2	3	3	2	2	1	2	2	1	1	3	2	6	4	5	3	2	5
																							15	
																							50	

April 5: CULTURE D

Starved 24 hours

I	Accepted..	2	3																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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These results from individuals are therefore strictly comparable and in accord with those obtained when a large number are tested at one time (table 1).

Now, in order to explain the cause of the change in reaction the suggestion might be offered that *Bursaria* shows a decrease in the rate of feeding because of the decrease in the amount of space in the body which food can occupy. This is undoubtedly true to some extent in those individuals which do not stop feeding until the cell becomes distorted by the comparatively immense mass of food. So far as the volume capacity of a normal individual of *Bursaria* is concerned, hundreds of observations have shown me beyond doubt that this may frequently be as much as *twenty-five to thirty grains*. Nevertheless, reversal of the cilia always takes place sooner or later. But the suggestion evidently does not apply to those individuals which show a change in the reaction when only a few grains have been eaten, for it seems impossible to understand how there could be a difference of as much as twenty grains of fresh yolk (table 2) in two normal individuals of equal size, when the cells are under exactly the same conditions, if this result were not due to a difference in the physiological state of the cells. Change in feeding was caused by the periodic reversal of the cilia and the reversal of the cilia in turn in some manner initiated or caused by a stimulus from the food already eaten, for it seems most natural to suppose that the stimulus originated from the change produced by the food mass in the interior of the cytoplasm. The most definite evidence that the change is due to stimulus from the eaten food is found in the radical change in the action of the cilia of the feeding mechanism.

If such fed individuals as those in table 3 are left in tap water free from food they may again eat yolk after digestion is partially or wholly completed, and again show a similar decrease in the rate of feeding, that is, a reversal of the oral cilia. The total quantity which will be eaten may be greater than that eaten at the previous feeding; but it usually is less, or often none at all.

The process of feeding in *Bursaria* shows it to be a functionally equilibrating system in its behavior towards food and the condition of its equilibrium at any particular time constitutes the physiological state which the cell is in, so far as its relation to food is concerned. The changes in the increase or decrease in the quantity of food eaten in successive meals and the increase or decrease in the rate of feeding might be discussed in the psychological terms 'hunger' and 'satiation;' but it is evident that the simpler terms quantity and rate express the facts of experiment, while any attempt at definitely determining whether the changes in quantity and rate are the same or different from 'hunger' and 'satiation' will obviously lead nowhere. Hence it seems better to use the terms, rate and quantity, which have a clear and quantitative meaning.

### *3. Other causes of individual variation*

*Bursaria* at times closes up its oral apparatus. This may take place to such an extent that the opening is smaller than the food particles and then the latter can of course not be eaten. This condition can readily be observed under the binocular and it can always be determined beforehand whether closure has taken place to such an extent that the organisms can not feed. Other minor accidental individual variations are also present to some extent. These may be partly due to the difference in the size of the grains of yolk eaten. Sometimes when an individual is weak, owing to prolonged starving or for some other reason, two or three grains may become stuck in the oral pouch and this prevents feeding until the animal succeeds in throwing them out or by other means they become loosened. The material used was always examined beforehand to make sure that it was in a healthy condition so that these accidental conditions play no part in the final results of the experiments described.

Such a series of experiments as the foregoing do not show us specifically what these complex conditions are which have been cloaked in the phrase 'physiological states.' This however is

not the object of the above experiments: they are only here considered for the purpose of demonstrating the existence of these conditions, the fact of change within them and especially in this connection their rôle in the *external* phenomena of feeding and food selection in *Bursaria*, and how they may affect the results which will be given in the following pages.

#### EXTERNAL RELATIONS OF THE FEEDING PROCESS

##### 1. *Effects of external factors on feeding*

*a. Concentration of the food supply.* The rate of feeding is within comparatively wide limits not dependant upon the concentration of the yolk suspension, provided it is not too low. This may be illustrated from one out of a series of experiments. The time of feeding was reduced to five minutes for the purpose of bringing out the effect of difference in the concentration more strongly. If the animals had been left in the suspensions twenty minutes (the usual time of feeding; cf. table 3) the difference would have been less marked, especially with material which shows a high rate of feeding.

*Experiment I.*<sup>5</sup> Material from a healthy culture was starved twenty-four hours in tap water. All were perfectly normal and active. The experiment was carried out in 8 cc. stender dishes. The concentration in dish *B* was 8 times that in dish *A*. Twenty individuals were placed in each dish. The results from trial number 2 represent more nearly the ideal because these two suspensions were kept uniformly distributed during the five minutes feeding, and the individuals were picked out alternately by fives. Both trials, however, express equally well the proportional effect of concentration, namely, 1 to 2, as compared to the proportion of concentration, 1 to 8<sup>6</sup> (table 4).

The concentrations used in the experiment are approximately represented by figure 3.

<sup>5</sup> The experiments given in this paper are numbered in regular order for the convenience of the reader, and do not represent the actual order. Only a small number of the experiments actually carried out are given.

<sup>6</sup> In all the experiments considered in this paper, where the concentration plays a part, the concentration was intermediate between those used in this experiment (fig. 3).

TABLE 4  
*Experiment I*

TRIAL	DISH	NUMBER OF YOLK GRAINS IN EACH INDIVIDUAL																				TOTAL	AVG. PER IND.
1	A	1	2	1	1	5	5	1	2	1	2	1	0	0	0	1	0	3	2	3	1	32	1.6
	B	1	2	6	3	7	4	4	7	5	1	2	0	2	3	1	4	3	3	1	3	62	3.1
SAME YOLK SUSPENSIONS USED																							
2	A	2	4	2	1	1	2	1	5	5	3	0	1	4	0	4	3	0	4	1	2	45	2.25
	B	1	8	6	1	4	4	3	8	4	1	5	7	6	7	2	4	1	0	7	2	81	4.05

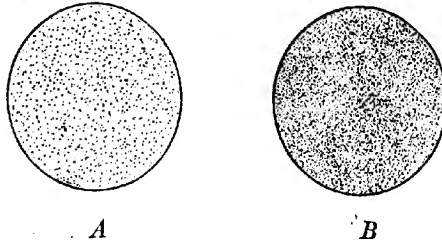


Fig. 3 Showing the relative concentration of yolk in dishes A and B of Experiment 1.

*b. Effect of mechanical stimulation and of mechanical injury on feeding.*

*Experiment II.* Thirty individuals from the same culture, starved twenty-four hours, were placed in each of six 8 cc. dishes containing 5 cc. tap water. Before feeding, the animals in three of the dishes (Set 1 in the experiment) were mechanically stimulated by means of a pipette. The opening of the latter was about ten times the width of Bursaria. The edges of the opening were made smooth by melting. The animals of Set 1 were stimulated by drawing them up into the pipette along with the tap water in the 8 cc. dishes, four times. Equal quantities of yolk suspension were now added to all the dishes. After having fed ten minutes the animals of Set 1 were again stimulated by drawing them along with the yolk suspension into the pipette two times; at the same time the control, Set 2, was stirred by gently shaking the dish and not allowing any instrument to touch the animals; hence the distribution of the yolk was the same in the two sets of dishes. All the individuals in Set 1, after having been stimulated, were perfectly normal and not injured. They looked like those of the

TABLE 5  
Experiment II  
SET 1. STIMULATED

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL	AVG. PER IND.		
																														grains		
A.....	0	1	4	0	1	0	1	3	1	5	1	1	2	0	1	1	0	1	1	1	0	2	2	0	1	3	0	0	0	0	33	1.1
B.....	1	0	0	2	0	0	0	0	2	0	0	0	2	0	0	0	2	0	0	3	0	0	1	0	1	0	0	0	1	1	17	0.56
C.....	4	0	0	1	2	1	2	2	6	0	1	1	1	3	3	1	1	1	1	3	0	0	4	0	3	0	0	3	3	48	1.60	
Total average.....																														1.06		

SET 2: CONTROL: NOT STIMULATED

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																											TOTAL	AVG. PER IND.			
																														grains		
A.....	3	5	7	2	3	6	3	3	3	4	14	13	3	1	2	5	8	2	9	5	9	5	10	3	7	7	6	3	4	2	157	5.23
B.....	2	1	3	6	3	1	5	1	3	6	4	4	5	2	3	2	5	6	11	4	2	6	6	1	3	2	7	2	3	0	109	3.63
C.....	6	1	3	6	5	6	5	3	2	7	4	4	4	3	3	6	5	4	3	2	6	1	3	2	3	5	6	2	2	1	113	3.76
Total average.....																												4.206				

control. If a smaller pipette is used or a larger one, and the stimulation, by sucking them along with the medium up into the pipette, is more violent, it will stimulate and injure the organisms so that they will not eat at all, or at least, not for some time after stimulation. Of course structural injuries are very easily produced, with the result that regulation of the cell must take place before any food can be eaten (table 5).

Proof that in this experiment, Set 1, if not in Set 1 of Experiment III, the organisms were not injured beyond the capacity for swallowing, is found in the fact that the great majority did eat, though only a comparatively small number of grains. Another experiment may be given to illustrate the same fact.

*Experiment III.* The animals in Set 1 were not stimulated before feeding, but after they had fed for five minutes they were stimulated by drawing the suspension with the animals in it, up into the pipette only once. Material from a different culture was used in this experiment; time of feeding fifteen minutes. The control suspension with the organisms was redistributed once by gently shaking the dish. The animals were all normal in form at the end of the experiment (table 6).

In Experiment III the stimulus was only slight as compared to that in Experiment II, yet the effect was marked. As stated above, strong stimulation may totally prevent feeding.

TABLE 6  
Experiment III  
SET 1: STIMULATED

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL	AVG. PER IND.		
																														grains		
A.....	5	4	1	6	8	6	9	0	7	0	10	3	0	1	3	3	4	0	1	4	6	5	1	5	0	0	0	4	0	0	96	3.2
B.....	0	0	4	0	4	15	11	0	0	9	0	1	4	6	5	2	5	8	4	0	9	6	6	11	4	5	6	0	2	0	127	4.0
C.....	5	3	0	4	0	2	1	3	5	0	3	1	0	1	0	0	4	2	0	3	1	3	3	0	8	0	0	3	0	4	59	1.86
Total average.....																														3.5		

SET 2: CONTROL: NOT STIMULATED

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL	AVG. PER IND.		
																															grains	
A.....	6	9	8	6	6	4	10	7	6	5	9	7	7	1	7	2	5	9	14	6	7	6	7	6	0	10	7	9	6	201	6.70	
B.....	6	2	4	6	6	1	7	9	5	4	6	4	6	2	7	1	6	10	0	4	7	2	5	5	5	6	7	10	6	3	152	5.06
C.....	0	11	4	6	6	9	8	6	6	9	6	5	3	9	7	7	9	2	7	4	7	8	5	7	12	6	8	6	9	1	198	6.60
Total average.....																													6.12			

The effect of mechanical stimulation must be emphasized because it shows that in any work of this nature it is necessary to handle the organisms gently. This relation must be inferred to apply to work on other Infusoria also, at least to some extent.

*c. Effect of temperature on feeding.*

*Experiment IV.* Thirty individuals starved twenty-four hours, were placed in each of six vials. Each vial contained 5 cc. of tap water. These vials were now placed in large dishes containing water kept at the desired temperatures. The latter were read on a small thermometer set inside of each vial. Equal quantities of fresh yolk suspension were added when the temperature had reached the desired point. They were fed fifteen minutes (table 7).

TABLE 7  
Experiment IV

		NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL		
deg. C.		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
5.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
10.....	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
15.....	2	0	1	3	1	3	4	2	1	0	0	0	0	6	4	0	3	5	1	0	2	3	1	0	1	0	0	6	4	0	4	52
20.....	9	12	4	19	6	9	8	2	0	6	2	13	14	6	5	9	2	0	4	2	4	5	4	8	7	9	6	7	5	8	195	
35.....	0	10	3	4	11	6	9	13	13	16	4	6	12	0	9	15	5	4	8	13	9	13	4	2	4	6	10	9	2	41	241	

39 }  
to } ..... All died in from 5 to 10 minutes  
40 }

The experiment was repeated with closely similar results. At lower temperatures the animals are always unable to eat. As the temperature is raised and the activity of the cell increases, the rate of feeding increases, continuing to increase nearly up to the point where the cell is injured or killed by the heat. At temperatures between 20° and 25°C. (i.e., at about the optimum) the increase in the rate of feeding can be determined only by using a very large number of individuals, since the variations obliterate the effects when a small number is used.

All the experiments relating to other conditions were carried on at temperatures ranging between 20° and 27°C. Where necessary (as in prolonged experiments on digestion) the temperature was kept constant to within 1° to 1.5°C., throughout the course of the experiment, by keeping the organisms in moist chambers in a constant temperature oven.

*d. Effect of HCl and NaOH on the feeding reaction.*

*Experiment V.* The medium used in this experiment (table 8) was conductivity water.<sup>7</sup> Any water less carefully purified is worthless for such experiments, as was shown by experiments carried out with tap water. By comparing the results it was strikingly evident that the acid and base had reacted with the salts and other impurities in the tap water and hence their effect was removed in low concentrations. The animals were washed once in conductivity water before putting them into the solutions. Time of feeding, twenty minutes (table 8).

It is seen from table 8 that the base NaOH was much more toxic than the HCl, and that as the concentration was increased the number of grains eaten became less and less. The chemical relations of the food and medium will be considered in more detail, later on (p. 29).

*e. Effect of strong white light on the feeding reaction.* Bursaria, when kept in dishes with a rather clear medium, often collect in the greatest number on the side of the dish away from fairly strong white light. It therefore became of interest to test what effect continuous light of a high intensity would have upon the rate of feeding.

<sup>7</sup> Prepared and used in the Department of Physical Chemistry for conductivity measurements.

TABLE 8.  
*Experiment V*

NaOH		TOTAL NUMBER GRAINS
MOL. CONC.	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL	
1/400.....	All dead in 3 minutes.....	0
1/600.....	All dead in 10 minutes.....	0
1/800.....	Many dead at 15 minutes; none eaten at end of 20 minutes.....	0
1/1200.....	All alive and normal in shape at end of ex- periment; no grains of yolk eaten.....	0
1/1600.....	0 1 0 0 0 1 1 0 2 1 2 2 0 1 3 2 0 3 0 0	19
1/3200.....	6 8 8 7 3 5 1 3 1 3 5 5 10 9 6 11 4 7 5 8	121
1/6400.....	5 9 8 8 6 14 8 1 11 5 8 5 12 4 8 11 6 8 6 5	148
CONTROL IN CONDUCTIVITY WATER		
	7 9 10 9 9 4 5 10 10 6 6 12 4 6 5 6 17 8 8 4	155
HCl		
1/400.....	All dead in 5 minutes.....	0
1/600.....	0 1 2 1 1 1 2 0 2 1 4 2 0 1 2 0 5 4 1 2	32
1/800.....	1 3 4 5 13 4 5 5 2 6 0 5 4 4 3 3 7 5 5 6	90
1/1200.....	6 8 1 8 7 8 6 0 6 3 8 3 1 5 4 1 1 2 11 1	90
1/1600.....	6 5 8 8 7 9 3 12 3 2 5 8 14 6 9 3 5 5 0 5	123
1/3200.....	8 7 15 5 11 13 8 9 0 2 4 1 3 8 2 10 5 5 6 8	130
1/6400.....	5 10 12 10 9 7 9 3 14 6 10 5 9 9 10 10 7 9 5 3	162
CONTROL IN CONDUCTIVITY WATER		
	12 12 4 13 0 11 14 5 6 2 0 9 7 8 2 11 3 11 3 4	137

*Experiments VI and VII.* White light from the arc of an Edinger apparatus was focused upon the stage so that a spot of light  $1\frac{1}{2}$  inches in diameter, of a very high intensity, was obtained. The light was filtered through a layer of water 1.5 cm. in thickness. An 8 cc. stender dish containing thirty normal individuals was placed in the spot of light and the usual quantity of yolk suspension added. A control was kept in weak diffuse daylight. The animals were fed twenty minutes. The following results show that continuous action of intense white light on the animals does not have any effect upon the rate of feeding. Two experiments with controls are given (tables 9 and 10).



TABLE 9  
*Experiment VI*

## STRONG WHITE LIGHT

NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
2	6	5	6	11	8	2	5	8	8	1	2	3	5	6	6	3	6	3	3	7
2	7	6	6	5	6	1	5	6	1	5	6									150

## CONTROL: DIFFUSE DAYLIGHT

8	5	6	4	3	6	0	10	0	5	4	4	1	2	4	3	3	7	3	6	7
7	8	6	7	4	7	9	2	5												146

TABLE 10  
*Experiment VII*

## STRONG WHITE LIGHT

NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
5	6	9	6	3	1	5	3	3	5	8	1	7	6	4	11	7	7	0	2	10
4	3	4	4	5	11	3	6	5												154

## CONTROL: DIFFUSE DAYLIGHT

8	8	2	5	3	10	9	11	8	9	2	7	8	1	0	4	15	0	8	2	8
10	6	7	6	8	11	3	4	5												188

*f. Effect of the electric current.* Weak induction currents have, within a limited time, no noticeable effect upon the feeding, as is shown by the following results from two separate experiments, VIII and IX. The total number of grains eaten by 20 individuals in each of two 8 cc. dishes is given in each experiment:<sup>3</sup>

*Experiment VIII*

Dish A—73, total number of grains eaten by 20 individuals

Dish B—69, total number of grains eaten by 20 individuals

*Experiment IX*

Dish A—65, total number of grains eaten by 20 individuals

Dish B—64, total number of grains eaten by 20 individuals

Control for Experiments VIII and IX; not stimulated by the current

Dish C—89, total number of grains eaten by 20 individuals

<sup>3</sup> The apparatus was arranged in such a way as completely to prevent any effect of substances liberated at the electrodes, by inserting the electrodes in a physiological normal NaCl solution in each of two 8 cc. dishes and from these the circuit was closed through the other two 8 cc. dishes containing the animals, by means, of small  $\Omega$  tube connections filled with tap water and plugged loosely with a wad of cotton.

When, however, a direct current is used of such strength that the organisms can be made to go to one side or the other by reversal of the current the effect becomes more or less apparent. Feeding can not be prolonged to twenty minutes with a strong direct current, for the organisms are easily injured. To obviate this, time of feeding was limited to five minutes. The animals were made to swim from one side to the other by frequent reversals of the current. In Experiment X, they were stimulated by frequent reversal of the direct current during the first minute of feeding and then left to feed four minutes more without stimulation. In Experiment XI they were stimulated in the same way during the whole period of feeding. Time of feeding, five minutes (tables 11 and 12).

TABLE 11  
*Experiment X*

STIMULATED BY DIRECT CURRENT, 1 MINUTE

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
																					grains
A.....	3	13	5	11	3	10	8	0	1	6	11	5	8	4	3	7	1	1	8	3	111
CONTROL: NO CURRENT																					
B.....	7	0	4	6	3	8	10	7	7	6	8	5	0	4	3	3	9	4	10	8	118

TABLE 12  
*Experiment XI*

STIMULATED BY DIRECT CURRENT, 5 MINUTES

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																			TOTAL	
																				grains	
A.....	1	3	4	6	2	3	10	6	5	10	5	0	0	2	3	8	7	1	3	11	90
CONTROL: NO CURRENT																					
B.....	5	5	11	0	2	18	3	1	11	0	11	13	10	0	5	7	0	9	5	6	122

As the results show, feeding was not discontinued under these conditions of strong stimulation by the current, though the or-

ganisms show a somewhat smaller total number of grains eaten than in the controls, in the same length of time. The difference is, however, too small to have a clear significance.

The strength of current may be increased but usually feeding can never be totally inhibited unless the organisms are injured or killed immediately after the yolk has been added.

The preceding experiments show clearly the relation which exists within certain limits, between the feeding reaction of this organism and a simultaneous reaction to certain other types of stimulation. During stimulation with HCl and NaOH, and especially with high temperatures and the electric current, the notable fact is that the reaction to food is strongly persistent under wide ranges of intensity of a second applied stimulus; this is true to such an extent that under some conditions the feeding continues up to the point where the intensity is so high that the stimulus is destructive to the organism. These facts must not be thought to be of general application, for evidently mechanical stimulation is quite effective in changing the reaction to food. What the behavior will be under two simultaneous stimuli obviously depends upon the nature of those stimuli.

It should be distinctly noted that in all the foregoing experiments the chemical as well as physical nature of the food substance has been kept constant while the organism in its particular physiological state has been acted upon by certain external agents; these being of a sufficient variety to indicate clearly what rôle these different types of factors play in the relation of this animal to food, and to serve as a guide to further inquiry.

We now have to see what changes are produced in the feeding reaction by modifying that factor which in the foregoing experiments has been kept constant,<sup>9</sup> namely, the food. In the following series of experiments all the other conditions will be kept constant, or at least arranged in such a way that they may be

<sup>9</sup> An exception to this might be taken in the experiments with HCl and NaOH for it is a question whether or not these affect the chemical character of the yolk sufficiently under the conditions of these experiments to modify the number of grains eaten. The yolk was not treated previous to the feeding; thus the time was so short and the dilutions so high that any change must have been very slight.

properly controlled and accounted for. We shall attempt to determine what the relation of Bursaria is, to specific physical and chemical properties of the food itself. First it will be determined how the external part of the reaction is modified, that is, what is the behavior of the cell in so far as this has to do with the selection of food.

#### SELECTION OF FOOD AND THE FACTORS CONCERNED

The object of the experiments described in the present section is to answer the question: Can Bursaria discriminate quantitative or qualitative differences between the yolk grains?

When fresh hard boiled yolk grains, prepared as described on page 8, are treated with different kinds of water-soluble dyes, the amount of dye which is adsorbed by a grain of yolk varies with the kind of dye used. At first a considerable number of different dyes in aqueous solution were tested in a comparatively rough way; first, for the relative amount of each dye which would be taken up by the grains of yolk; second, for the rate at which the dyes were adsorbed and the ease with which they could be washed out (reversibility of the adsorption); and third, for the relative toxicity of aqueous solutions of these dyes to the organisms. Among the dyes so tested were fuchsin, lyons blue, methylin blue, eosin, cyanin, gentian violet, saffranin, janus green, congo red, and an aqueous solution of hematoxylin.

The results of the following experiments on food selection, in so far as they are related to the dye, depend upon the three factors named: (1) The amount of dye adsorbed (2) The rate of the reversible adsorption reaction, and (3) The relative toxicity to Bursaria, of the dye in aqueous solution.

It was quickly found that certain dyes were better suited than others, for the particular end in view. Aqueous solutions of saffranin and janus green were found best to fulfil the necessary conditions. Both show a reversible adsorption with yolk, while the velocity of the reversible adsorption is sufficiently low to prevent a too rapid washing out of the stain. By this means one is able to control the amount of adsorbed dye much more

easily than if it could be washed out quickly, and one is also able to control the concentration gradient between pure water and the dye adsorbed by the yolk grain. The toxicity of the different dyes varies greatly, and it was found that saffranin and janus green were best from this point of view also, since both of these are very toxic to *Bursaria* in higher concentrations but only slightly so in lower concentrations.

### 1. Experiments with stained and unstained yolk

#### a. Saffranin.

*Experiment XII (a).* Object, to test (a) whether or not *Bursaria* will eat yolk grains which have adsorbed an appreciable amount of the soluble toxic substance saffranin and (b) whether or not the amount of yolk eaten depends, in this experiment, upon the amount of saffranin adsorbed.

Equal volumes of a strong suspension of fresh yolk were placed in each of seven stender dishes of 8 cc. capacity. A bright rose-colored solution of saffranin was made up with tap water. To the dishes designated A, B, C, D, E and F was added 5, 4, 3, 2, 1, and 0.5 cc. respectively, of this solution, and mixed thoroughly. The seventh dish without stain, was kept as a control. The suspensions were left to settle five minutes, then decanted and 5 cc. tap water added to all the dishes; this was repeated three times. The organisms used were starved twenty-four hours and were in excellent condition. The time of feeding was fifteen minutes (table 13)

TABLE 13  
*Experiments XII (a)*

DISH	SAFFRANIN SOL. C. C.	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
		grains																				
A.....	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	5
B.....	4	0	0	0	0	4	5	0	3	1	0	0	0	0	1	3	0	1	0	0	0	34
C.....	3	3	0	6	0	0	2	0	8	3	1	2	8	4	2	4	0	1	0	0	2	81
D.....	2	2	0	0	3	1	1	0	2	4	2	1	2	2	1	3	3	2	0	6	5	56
E.....	1	4	3	1	2	7	6	2	1	7	9	0	3	7	2	0	12	6	4	0	4	113
F.....	1/2	4	6	11	6	1	2	4	0	3	0	3	9	3	4	3	6	5	4	5	9	138
Control G.....	0	0	5	6	6	7	4	9	12	4	9	9	11	4	2	7	7	0	6	12	1	188

When stronger solutions of saffranin than that in A were used, no grains were eaten. All the animals at the end of the experiment were normal and had not been injured. The yolk of dish

### Experiment XII (b)

This indicates that we may obtain the same result whether we proceed with a strongly stained yolk and test successively after each washing, or, as in the former experiment, by staining different portions of yolk to different degrees to begin with. This was actually done in other experiments not given, and results exactly similar to those in Experiment XII (a) were obtained.

TABLE 14

### Experiment XIII

This shows that they were not injured sufficiently in even this strongly stained suspension totally to prevent them from eating unstained yolk immediately afterward. The cause of most of the 0's in the count is that, as the toxicity of the solution increases, the organisms have a tendency to close up the oral apparatus and do not open it again sufficiently, within the next twenty minutes or so, to be able to take in the yolk grains. Of

course if yolk which has been very strongly stained and not washed out sufficiently, is fed, then the concentration of the medium rises so quickly that they are greatly injured or killed.

Now against the conclusions which will be drawn from the results of Experiment XII (a) and (b), as it stands, may still be urged the objection that the reason that so few or no grains are eaten, is because of what one might call a general injury or stimulation of the cell by the saffranin which is rapidly being liberated into the water, and that it may not have anything to do with a specific reaction to the chemical character of the food particle as such, that is, to anything like a "sense of taste." That this objection does not apply to conditions like those in the above experiment (XII, a and b) where the amount of stain adsorbed even in dishes A and B is very little compared to that in Experiment XIII, may be shown by taking the solution of dish A, Experiment XII (a), and placing unstained yolk and Bursariae in it. The result of such an experiment is that the organisms fill up with fresh yolk, showing that the medium in weaker concentrations does not affect the eating process to any appreciable extent. \* Further proof of this will be given in the experiments immediately following, and also in experiments to be given later.

*Experiment XIV.* To test whether or not Bursaria can select and eat non-toxic yolk grains from among toxic ones, when the two are mixed. Two suspensions were made, one of yolk stained in saffranin twenty-four hours, then washed out repeatedly, the other containing the same kind of yolk washed in the same way but not stained. The two yolk suspensions were mixed immediately before the animals were placed in the mixture. Twenty individuals were used. The time of feeding was fifteen minutes; a control of washed unstained yolk alone, was kept at the same time (table 15).

TABLE  
*Experiment XIV*

MIXTURE	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																		TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	0	5	2	3	2	1	2	4	2	0	1	2	3	2	0	0	0	0	29
Control:																			
Unstained yolk .....	12	16	7	15	4	9	15	8	9	9	16	3	25	7	15	12	9	9	213

In the mixture the concentration of the saffranin rose so rapidly that some of the individuals were killed.<sup>10</sup> This is indicated above by X. Yet even in such a strong solution, selection took place, though the number of grains eaten was small compared to the number in the control.

*Experiment XV.* Another sample of yolk less deeply stained than that in Experiment XIV, was washed out many times and mixed with an equal quantity of unstained yolk from the same sample. Thirty individuals of the same material as used in Experiment XIV, were fed for five minutes, instead of fifteen minutes as before (table 16).

TABLE 16  
*Experiment XV*

MIXTURE	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																								TOTAL	
Stained.....	1	1	1	0	1	1	0	3	3	2	2	0	1	0	3	1	2	4	4	1	2	2	1	2	0	43
Unstained.....	2	6	7	2	16	14	4	1	1	1	1	2	7	13	9	0	6	5	12	9	5	7	2	10	7	216

A repetition of the above experiment with yolk stained a little more deeply gave the following result; twenty individuals used; fed five minutes (table 17).

TABLE 17

MIXTURE	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	3	1	2	2	1	0	1	1	1	1	2	1	3	0	1	3	2	1	0	25
Unstained.....	4	0	0	1	4	5	5	0	4	2	3	5	3	0	1	3	0	12	1	3	56

The control of Experiment XIV will likewise serve for Experiment XV.

The results of this experiment are to be explained by the fact that the concentration gradient of the adsorbed toxic saffranin is relatively low with respect to the gradient of the water-soluble yolk substance to which *Bursaria* reacts in a strongly positive manner. Of course one is not to suppose that it is the relative molecular concentration gradient alone that determines the re-

<sup>10</sup> The cytolytic action of saffranin is in some ways more marked than that of janus green. The character of its reversible adsorption reaction also makes it less suited for use in experiments of this kind than janus green, as will appear from results with the latter.



sult. What is meant in this case by concentration gradient, is the molecular concentration plus the specificity of the substance, that is, in anthropomorphic terms we should say "the kind of taste" which the substance has. That the specific nature of the substance is one factor in determining the result, is shown by a comparison of the results of numerous experiments with saffranin, janus green, hematoxylin, and especially other less toxic stains, like congo red (cf. what follows).

*b. Janus green.* A considerable number of experiments have been carried out using this substance, with the same general results as those obtained with saffranin. It is better adapted to bring out the phenomenon of selection than saffranin, causing a sharp discrimination by *Bursaria*; small quantities adsorbed by the grains are sufficient to bring about rejection. The following experiments show some of the relations.

*Experiment XVI (a) and (b).* Yolk was stained in janus green twenty-four hours then soaked in tap water and washed repeatedly. A portion of the same kind of yolk soaked and washed in the same way but not stained, was used as a control and for mixing with the stained yolk. A few minutes before the experiment equal quantities of the stained and unstained yolk suspension were mixed in dish A. A second quantity of the unstained yolk suspension of a concentration equal to the sum of those in dish A was placed in dish B. Twenty individuals were placed in each dish and left to feed twenty minutes (table 18 a).

TABLE 18 (a)

*Experiment XVI (a)*

MIXTURE, DISH A	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																			TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	5	3	1	6	2	4	1	4	5	1	5	1	4	4	1	1	1	1	1	47
Control,																				
Dish B:																				
Unstained.....	9	12	21	8	20	12	15	19	13	17	14	13	15	12	19	17	14	7	8	268

The yolk in both dishes was now washed twice and the experiment repeated with control. Time of feeding fifteen minutes. The count is shown in table 18 b.

In (a) the solution had become sufficiently strong to affect five of the animals (X), so that they could not be recovered for

TABLE 18 (b)  
*Experiment XVI (b)*

MIXTURE, DISH A	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	0	0	0	1	0	0	1	1	2	0	1	2	2	4	0	0	1	0	1	3	19
Control, Dish B:																					
Unstained.....	16	8	17	7	13	10	11	12	5	12	4	5	12	15	15	16	7	10	13	8	216

the count. The smaller number of grains eaten in (b) by those in dish A is due in part to the shorter time of feeding but more to the fact that the unstained yolk grains had by this time adsorbed some of the liberated janus green from the stained yolk grains (see Experiment XVIII, p. 36).

*Experiment XVII (a) and (b).* The results of this experiment are given to show that Bursariae from two different cultures may show different reactions in selection experiments. In part (a) material was used from one wild culture, while in part (b) material from a different one was used. Both were starved twenty-four hours before using them. All other conditions were alike. Time of feeding fifteen minutes (table 19).

TABLE 19  
*Experiment XVII*

MIXTURE, DISH A	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	0	4	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	9
Control: dish B,																					(a)
Unstained.....	5	4	6	2	4	7	6	3	9	3	8	3	3	6	4	3	2	7	6	8	99
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	3	4	0	2	10	3	3	2	7	6	1	6	6	0	8	2	1	0	5	1	70
Control: dish B,																					(b)
Unstained.....	2	4	13	9	5	7	9	2	4	10	6	15	9	13	6	11	9	1	6	7	148

In such experiments as these it was found that occasionally an individual had eaten a stained grain along with the unstained ones, but this happened very seldom in any of the experiments with janus green.

*Experiment XVIII.* The two dishes A and B in Experiment XVII (b) were left standing for two hours; then they were washed once and tested with the same material used in Experiment XVII (b), in order to show the effect of the adsorption of the liberated stain by the unstained grains mixed with the stained ones. Time of feeding fifteen minutes (table 20).

TABLE 20  
*Experiment XVIII*

MIXTURE, DISH A	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	0	2	0	7	2	0	1	4	0	1	0	0	0	2	0	0	0
Control: dish B,																	
Unstained.....	18	23	8	15	13	7	0	9	12	15	4	17	16	12	15	15	11
																0	810
																	228

This shows that when the janus green yolk is left with the unstained yolk for some time, the liberated stain from the janus green yolk is adsorbed by the unstained yolk grains, and as a result the latter are not eaten so readily. If the mixture is left standing too long and then rinsed in tap water, then no grains are eaten. Bursaria can react to such small quantities of adsorbed janus green that the amount adsorbed cannot be distinguished by the eye, when the unstained yolk grains mixed with the stained ones are examined.

*Experiment XIX.* To prove that the solution of the janus green which is produced by the liberation of the stain from the stained yolk, does not even in quite strong concentrations prevent the eating of fresh yolk placed in it, the result of one test out of a considerable number made at different times, is given. A solution was drawn off from janus green stained yolk used in an experiment in which no grains of the mixture had been eaten, after standing for some time. To this solution was added unstained yolk. Ten individuals were tested (table 21).

TABLE 21  
*Experiment XIX*

	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL										TOTAL
Fresh yolk in janus green solution, dish A.....	8	3	6	3	1	0	3	0	3	0	27
Control, fresh yolk in tap water, dish B.....	5	15	4	5	0	6	5	3	1	0	44

It is evident that the solution of janus green drawn from the mixed suspension, and produced by the liberation of the stain from the stained yolk grains of the mixture which was not eaten, did not now prevent the animals from eating unstained yolk grains which were placed in it; hence it was not the stain in solution which prevented the eating of the stained grains of the mixture from which the solution was drawn; but the stain which was adsorbed by the yolk grains of the mixture and diffused from them. Many such similar tests were carried out giving the same result. This does not mean that the solution apart from the yolk grain with its adsorbed dye, may not affect the result of the feeding, for in higher concentrations the solution apart from the stain upon the grain does affect the feeding process. In solutions of lower concentrations of the appropriate dye the chemical nature of the grain along with the amount of dye adsorbed, are the essential factors determining the number of grains which will be eaten.

*c. Hematoxylin.* To show further that the specificity of the toxic agent plays a large part in determining whether or not yolk will be eaten, the following experiments are given. It will be noted that in this case we have a substance which has a very different effect upon the cell and its relation to food, from that produced by the substances thus far dealt with. The solution in this case may be made very deep brown while the grains are also stained deeply, and yet the yolk grains are eaten even in solutions which kill the animals if they remain in it more than three or four minutes.

*Experiment XX. Table 22 (a) and (b).* The same quantity of yolk was added to each of nine dishes of 8 cc. capacity, each containing equal amounts of tap water. The dishes were numbered 1, 2, 3, and so forth. To these were added diverse quantities of the 0.5 per cent aqueous solution of hematoxylin by drops, as given in the tables; time of feeding ten minutes.

This experiment was repeated with the same suspensions at the end of one hour; time of feeding fifteen minutes (table 22 b). The individuals which died before the count was made are denoted by X. The tables show that although the solutions, espe-

cially in higher concentration, are very injurious, the organisms, nevertheless, eat the grains of yolk. After one or more hours the grains become stained deeply. This was the case in table 22 (b). The increase in the length of time of feeding (i.e., the time the animals were left in the solution) is the cause of the high mortality in table 22 (b).

TABLE 22 (a)  
Experiment XX

NUMBER OF DISH	1	2	3	4	5	6	7	8	9
Number of drops of $\frac{1}{2}$ per cent aq. hematoxylin.....	4	8	12	16	20	24	28	50	0
	0	0	0	2		1	4	7	7
	4	1	2	3		4	6	0	6
	6	2	0	11		7	3	×	10
Number of grains eaten by each individual.....	2	5	4	1		3	7	×	11
	2	4	0	1	lost	1	1	×	13
	0	0	0	1		11	1	×	8
	4	1	1	2		7	4	×	14
	6	7	3	4		0	4	×	6
	0	3	0	13		2	2	×	6
	4	0	7	×		4	×	×	8
Total.....	28	23	17	38+		40	32+	(7+)	89

TABLE 22 (b)

NUMBER OF DISH	1	2	3	4	5	6	7	8	9
Number of drops of $\frac{1}{2}$ per cent aq. hematoxylin.....	4	8	12	16	20	24	28	50	0
	3	1	0	1	1	0	×	×	11
	5	1	1	2	×	3	×	×	8
	0	2	0	1	×	0	×	×	7
	3	3	4	0	×	2	×	×	11
Number of grains eaten by each individual.....	2	0	3	0	×	×	×	×	14
	5	6	0	0	×	×	×	×	8
	4	0	0	×	×	×	×	×	6
	5	3	0	×	×	×	×	×	8
	3	1	0	×	×	×	×	×	13
	3	×	×	×	×	×	×	×	7
Total.....	33	17+	8+	4+	1+	5+	-	-	93

That the yolk grains are eaten, though to a less extent, even after having been left in the solution of 50 drop concentration for three and one-half hours, is shown by the following: Feeding was limited to three minutes, which in part accounts for the comparatively small number of grains eaten. Ten individuals were used. The count gave 4, 2, 1, 6, 1, 1, 2, 0, 0, 1, a total of 18 grains of the deeply stained yolk. When this deeply stained yolk in the 50 drop concentration of hematoxylin was washed four times in tap water and tested again; the following count was made: 11, 13, 14, 6, 5, 2, 12, 2, 4, 5, a total of 74 grains.

These tests show (a) that although a dye may be toxic to Bursaria, it may nevertheless not affect, to any great extent, the functioning of the feeding mechanism in the taking in and swallowing of the food, though (b) with some dyes total rejection of the food may take place, when the concentration is so low that it has only a comparatively slight cytolytic effect. The former condition is shown to a less marked extent in the experiments with saffranin than in the experiments with hematoxylin; while the latter condition is illustrated by the results with janus green. This seems then also to strongly suggest that different substances may affect different parts of the cell differently. Corroborative evidence upon this point, which it would be out of place to consider here, has been obtained from observations showing that the localization of the beginnings of cytolysis of the cell body of Bursaria may differ with the particular nature of the toxic agent employed.

*d. Congo red.* Another stain which is adsorbed readily is congo red. This, however, unlike hematoxylin, can only be washed out in part, that is, its adsorption reaction is not completely reversible. Also since this dye is not as toxic as saffranin or janus green, a large quantity of the stain may be adsorbed and yet not appreciably affect the number of grains eaten, as is shown by the following experiment.

*Experiment XXI.* The yolk was stained twenty minutes in a strong aqueous solution of the dye. Time of feeding twenty minutes. Thirty were used (table 23).

TABLE 23  
*Experiment XXI*

NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																											TOTAL				
Congo red, dish A																															
Stained.....	7	3	0	1	7	6	2	5	1	2	5	4	4	3	3	2	4	4	4	0	5	1	7	3	2	4	6	3	5	4	115
Control dish B																															
Unstained.....	11	8	7	3	6	8	5	6	6	5	8	7	5	1	2	5	6	7	1	5	8	6	7	10	5	6	5	9	4	7	179

In this case we have a comparatively low concentration gradient of the dye, together with a low toxicity and hence the comparatively small difference in the readiness with which *Bursaria* eats the stained and unstained yolk.

*e. Sudan III.* To show that an adsorbed substance which is insoluble in the medium has no determining effect upon the feeding and food selection, Sudan III was employed. This substance is insoluble in water but soluble in ethyl alcohol and fats.

*Experiment XXII.* Fresh yolk was stained in an 80 per cent alcoholic solution of Sudan III for a short time. It was then dried in an oven at 27°C. for twenty-four hours. A control of fresh yolk was also kept. The organisms were fed twenty minutes. The yolk takes on a very deep color with this stain.

TABLE 24  
*Experiment XXII*

	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																								TOTAL
Sudan III, stained yolk.....	13	18	19	0	15	3	5	7	18	25	15	5	7	8	9	17	18	12	3	26					243
Control, unstained..	18	3	4	14	7	15	17	22	6	28	20	6	9	3	29	18	13	10	8	24					274

It is evident that the insoluble Sudan III had no appreciable effect upon the food reaction. Mixtures of these showed no difference in the amounts of the two kinds of yolk eaten.

*f. Stale yolk.*

*Experiment XXIII.* Mixtures of fresh and stale yolk could not be used since the grains of the two kinds of yolk were visibly indistinguishable. One experiment is given. The stale yolk was four weeks old while the control was freshly prepared; both were of the same concentration (table 25).

TABLE 25

*Experiment XXIII*

	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																			TOTAL	
Stale yolk.....	0	2	5	1	0	3	8	2	1	1	4	0	6	1	5	1	3	1	1	3	48
Control, fresh yolk.	8	4	7	2	1	1	4	4	9	3	0	7	9	5	2	2	6	1	0	1	96

A difference is plainly evident. Other experiments show greater or less difference, depending upon the conditions.

2. *The basis for and nature of food selection in Bursaria, as shown by the foregoing and other experiments*

It must be remembered that in any such experiments as the foregoing the relation to food is in some ways an entirely new one to the organism. Yet it must be insisted upon that the yolk used in these experiments is assimilable by the organisms (a fact which will be considered at length in a later paper) and especially that whatever the mechanism of feeding and selection in nature is, it must be the same one which is brought into action in these experiments. Hence the criticism imagined above would appear to have no importance for the question under consideration here. In fact, it is to be believed that so far as these experiments are concerned, they are only a more strongly emphasized condition of what we find in nature and that they picture to us, so far as they go, the actual condition of the food relation of Bursaria in its native culture.

We may state the results briefly in the following way: First: Yolk grains are rejected if the soluble adsorbed toxic substance makes with the medium a sufficiently steep concentration gradient. If this gradient is low relative to that of the yolk-soluble substance, to which Bursaria reacts positively, then the organism may eat the stained yolk, other conditions being equal. Second: (a) Whether Bursaria will eat stained yolk grains or reject them depends also, along with the steepness of the concentration gradient, upon the specific chemical properties of the adsorbed sub-



stance in question, and furthermore (b) the substance by virtue of its chemical properties has, at least in some cases, a specific action upon the mechanism of feeding and selection, as is shown by a comparison of the results of the experiments with hematoxylin, saffranin and janus green. Additional evidence obtained from observations upon the phenomena of cytolysis in *Bursaria* also points to the correctness of this conclusion. A familiar instance of a similar nature is the casting off of the peristome by *Stentor* when stimulated or injured by chemicals. Another instance is the fact found by Jennings that the anterior end in *Paramecium* is more sensitive to mechanical stimulation than are other parts of the body.

That in feeding experiments with the Protozoa it is difficult to discriminate closely between the effects of the medium and those of the food substance itself is obvious, since (a) the amount eaten depends upon so many factors other than the nature of food and (b), since the organism selects on a chemical basis, which involves a soluble substance or substances diffusing into the medium from the food particle, hence necessarily involving the external medium to a greater or less extent. It is of course clear that differences in certain physical characters of food may likewise determine whether or not it will be eaten. This is shown most simply by objects which are too large, such as large yolk grains and large individuals of *Stentor*, which cannot be swallowed.

From all the facts found from experiments upon food selection by *Bursaria*, there is no evidence that active selection is based upon either "size, weight, form or surface texture" or any combination of these, except in so far as simple mechanical conditions would make them effective. All the facts show clearly that the chemical nature of the food is the property upon which the power of discrimination by *Bursaria* depends. Hence I find no evidence from *Bursaria* to support Schaeffer's contention that "*Stentor* selects its food upon a tactual basis and apparently not upon a chemical one" and that "*Stentor* reacts in selecting food, to physical properties only or chiefly, and not to chemical properties" (Schaeffer '10, page 131). On the other hand, the

facts which have been found in this connection are in agreement with the results and conclusions so far as they have been worked out by Metalnikow ('12) for *Paramecium*.

#### THE RELATION OF BURSARIA TO DIGESTIBLE AND NON-DIGESTIBLE SUBSTANCES

##### 1. *The external relations*

Many substances which are in the ordinary sense chemically indifferent to the organism are likewise eaten, though generally in small quantities. Among these are cinnabar, carbon black, chinese ink, powdered aluminium and the like. The relation of *Bursaria* to this class of substances is however strikingly different inside of the cell and to a large extent outside, when compared to that relation in the case of digestible and assimilable ones. The fact that some comparatively indifferent substances like the above, are eaten does not affect our conclusion drawn above, as to the paramount importance of the chemical properties of the food in food selection. Chinese ink contains some mucilaginous matter which as my own observations have shown me, is reacted to positively by *Bursaria* and hence the ink is quite readily eaten. Carmine is a similar substance which though generally taken to be insoluble in water, is in fact sufficiently soluble clearly to affect the feeding reactions of *Bursaria*. Furthermore, the fact that a substance may be insoluble does not, of course, prove that the stimulus from it is not a chemical one, for it is probable, that with such substances as aluminium, catalytic or other specific chemical, or even physical reactions dependent upon the chemical properties of the substance, are produced by contact with the plasma membranes. The possible variety of interactions of the cell with different kinds of substances when considered in this order of magnitude may of course be very large.

As regards the eating of non-digestible substances, powdered aluminium may serve, in one way, to illustrate the external relations. If a large number of individuals are put into a suspension of aluminium, often few if any will eat any of the particles of

aluminium and those that do eat it generally take in only a small quantity. This is also true of Sudan III and of carbon black. The quantity eaten varies with the conditions in a similar way, as previously set forth for yolk. Now if fresh yolk grains are added to the suspension of aluminium the animals will often quickly fill up with yolk, but in this case flakes of aluminium become attached to the yolk particles and hence often considerable quantities of the metallic aluminium are passed into the body along with the yolk. Sometimes the quantity of yolk eaten in such a mixed suspension is less than that in the control. This serves to illustrate the sort of equilibrium which exists between the organism and the kinds of substances in suspension, partly determining the amount of food and other substances eaten.

## 2. *The internal relations*

It was interesting to find that *Bursaria* possesses what I shall call an internal compensating reaction to those substances which are eaten to some extent, but are not digestible, such as Sudan III, chinese ink, powdered aluminium, and so forth. This compensating reaction makes up to some extent, in the "economy of the organism" for the lack of a perfect discrimination between indigestible ('tasteless') substances and those which can serve as food. It is shown by the fact that indigestible substances are eliminated from the cell usually a long time before the digestion of a similar quantity of food is completed. This may be shown with Sudan III. The results of the experiments are, for the sake of brevity, given by curves.

*Experiment XXIV: Figure 4.* Three sets of twenty-four individuals each were fed Sudan III, cold-ether-extracted yolk, and fresh yolk respectively. They were placed two in each watchglass containing tap water in moist chambers, and examination in this case was made at the end of three, seven, and twenty-two hours. Points on the abscissae indicate the length of time in hours after feeding, while points on the ordinates show the number of individuals which had extruded Sudan III (curve A) in the time intervals between the examinations, or in the case of extracted yolk (curve B) and fresh yolk (curve C) the number of individuals that had lost all traces of food. In this experiment the observations were not sufficiently frequent to bring out the actual

# RELATION OF BURSARIA TO FOOD

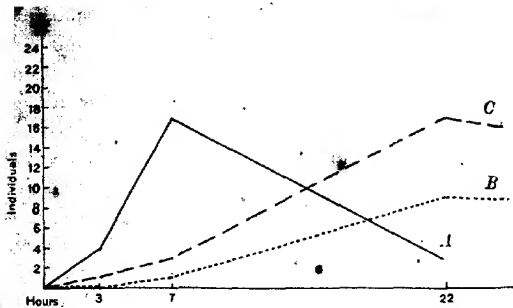


Fig. 4. Experiment XXIV. Curve A represents the course of total extrusion of Sudan III; curve B, that of complete digestion of cold-ether extracted yolk; curve C, course of complete disappearance of fresh fat-containing yolk.

course of the extrusion of Sudan III or of the disappearance of the yolk from the cytoplasm, but it will be noted that the great difference appears in the observation at the end of seven hours. At the end of twenty-two hours A had no traces of Sudan III, B still had six and C had ten individuals with food.

This early extrusion of indigestible substances is considered in more detail in connection with chinese ink in the following.<sup>11</sup>

*Experiment XXV.* Two sets of forty-eight individuals each were fed, one with cold-ether-extracted yolk, the other with chinese ink. Examination of the cell content was made at hour intervals as indicated by numbers on the basal abscissa. Ordinates indicate the number of individuals which had extruded all the ink content in the time indicated (curve A). In curve B the ordinates represent the number of individuals fed extracted yolk in which yolk had disappeared at the end of the time indicated by the abscissa. Chinese ink in suspension is much more readily eaten than carbon or aluminium and is therefore more convenient. This is to be explained by the fact that there are present mucilaginous soluble substances in the chinese ink which serve as agents inducing a more positive feeding reaction and are possibly of some slight food value. The ink was not found to be injurious to the animals. The greater part of the ink is thrown out quite early while slight traces may remain for some time longer. The time during which the ink was retained was taken to end when the last trace

<sup>11</sup> In order to obtain satisfactory results with such substances as Sudan III and aluminium in aqueous suspension the adsorbed gases should be driven off before feeding.

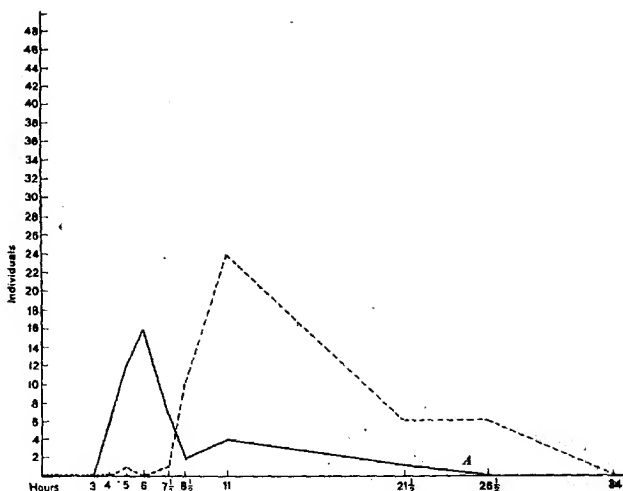


Fig. 5. Experiment XXV. Curve A represents the course of extrusion of chinese ink by forty-eight individuals; curve B that of complete digestion of a similar quantity of cold-ether extracted yolk by another set of forty-eight individuals from the same culture.

had been eliminated; curve A does not therefore represent the actual time at which the greater part of the ink was extruded but should have its maxima farther to the left than shown. This statement applies to all the extrusion curves which are given.

In figure 5 curve B, is that of complete digestion; no extrusion of the extracted yolk took place in this experiment.

After it had been thus shown that ink fed alone to one set of individuals was extruded long before digestion is completed of a similar amount of extracted yolk fed to another set, experiments were carried out to test what the reaction would be if both ink and extracted yolk were fed to the same individuals at the same time. The following two experiments are given to bring out the facts in a quantitative way.

*Experiment XXVI.* Fifty-four individuals were first fed chinese ink and immediately afterwards fed with cold-ether-extracted yolk. Two individuals were placed in each watch-glass containing 5 cc. of tap

water and kept in moist chambers. Records were taken noting the presence or total absence of ink and presence or completion of digestion of the extracted yolk at one hour intervals beginning with three and one-half hours up to twelve hours after feeding; three more records were taken at twenty-four, thirty-three and forty-eight hours. The results are expressed in curves in figure 6. Curve *A* represents the extrusion of ink; curve *B*, that of complete digestion of yolk.

It is seen from the relation of the curves that even in this case the ink is extruded before digestion of the extracted yolk is complete, provided that a sufficient quantity of yolk has been eaten.

It was noted that a short time after the ink had been eaten it became assembled into one or several rather definite lumps. This takes place before extrusion. Closer observation further revealed the fact that when ink particles came to be included in vacuoles containing yolk they were not extruded until the food of those vacuoles had been digested, while those which were not included by the yolk vacuoles were very soon extruded. This fact can readily be made out while one follows such experiments as Experiment XXVI above. Bursaria therefore has a power of simultaneous selective extrusion of the contents of different vacuoles as well as a power of selection in the feeding process. This mechanism obviously compensates for the lack of a perfect discriminative and selective function of the oral apparatus.

The results of an experiment (fig. 7) where these facts were taken into account for the purpose of expressing them in a graphic way in curves, is given in the following experiment. A control for comparison was also kept in this case (fig. 7, curve *C*).

*Experiment XXVII.* Forty-eight individuals were used in each of both the experiment and control. The control (curve *C*, fig. 7) which was fed ink only, shows a sharp early maximum of extrusion from five and one-half to seven and one-half hours after feeding, with three or four individuals retaining traces of ink as long as ten and one-half to twelve hours. Curve *A* represents the extrusion of ink in the forty-eight individuals fed both ink and extracted yolk. It shows two maxima exactly similar to those of curve *A* in Experiment XXVI. Curve *B* (fig. 7) represents the course of complete digestion of the yolk in the same individuals as those of curve *A*. There is only one maximum

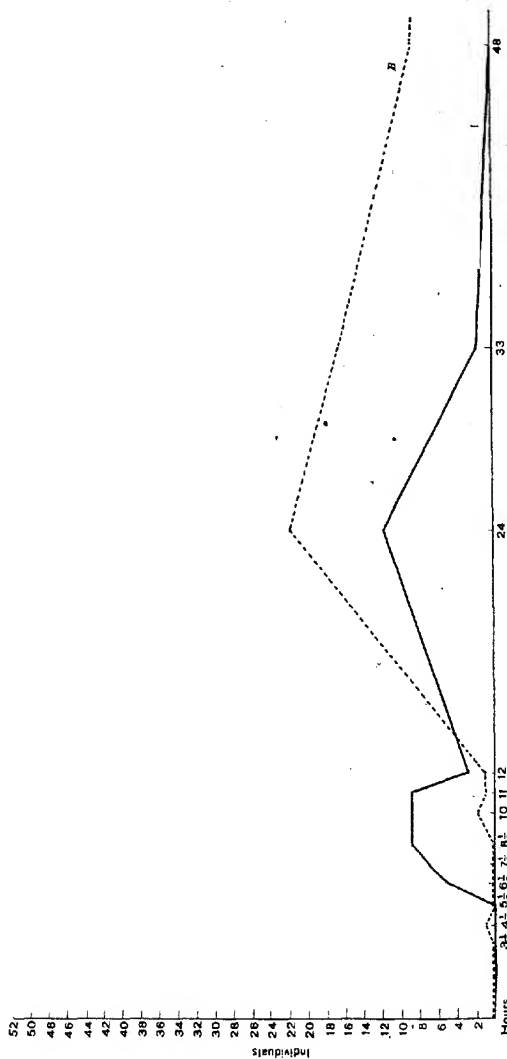


Fig. 6 Experiment XXVI. Curve A represents the course of complete extrusion of Chinese ink fed to fifty-four individuals; it shows two maxima; curve B, course of complete digestion by the same individuals as of curve A; curve B has only one maximum.

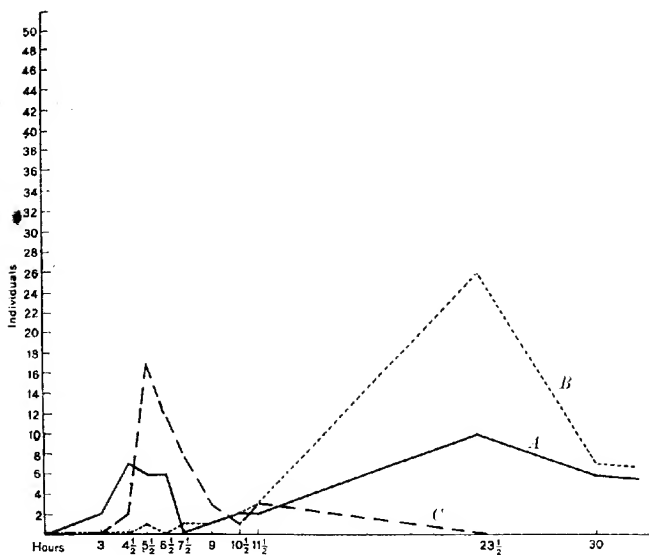


Fig. 7 Experiment XXVII. Curve A, course of extrusion of Chinese ink showing two maxima (cf. fig. 6, curve A); curve B, that of complete digestion, by the same individuals used in curve A, of extracted yolk; curve C, control: course of extrusion of ink by forty-eight individuals fed ink alone.

in this curve, and this comes about eighteen hours later than the first maximum of curve A and at about the same time as the second maximum of B. The significance of the second maximum in curve A is brought out in the following analysis of the two curves A and B, that is, in a quantitative analysis of the reactions of the forty-eight individuals used in the experiment. All the individuals which at any of the examinations had ink and yolk present in the same vacuole, or vacuoles, were recorded, hence we have a means of dividing the forty-eight individuals into two groups. Group I is made up of those in which, throughout the experiment, yolk and ink were in distinct and separate vacuoles, while Group II, includes those which had during part or all of the time one or more vacuoles which contained both ink and yolk in the same vacuole or vacuoles. Now we have the data which will show just what relation ink and yolk have to each other in the cytoplasm of Bursaria, and what the reaction of the cell is toward each of the two conditions represented by the composition of the vacuoles of the two Groups I and II.



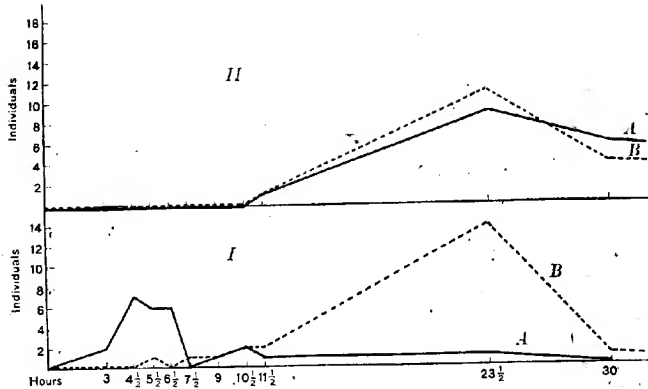


Fig. 8 Analysis of curves A and B of Experiment XXVII, figure 7. *I*, curve A, course of complete extrusion of ink from vacuoles containing only ink; curve B, course of complete digestion of extracted yolk in vacuoles containing only yolk, of the same individuals used in curve A. *II*, Curve A, course of complete extrusion of ink from vacuoles containing both ink and extracted yolk; curve B, course of complete digestion of extracted yolk from the same vacuoles in the same individuals as in curve A.

We may plot the curve of extrusion of ink in group I, and the curve of complete digestion of the same individuals. The curves are given in figure 8, *I*. The same was likewise done for Group II (fig. 8, *II*).

Curve A of figure 8, *I*, (ink and yolk in separate vacuoles) shows now only one early extrusion maximum instead of two. Curve B of figure 8, *I*, is lower (owing to the smaller number of individuals) but exactly similar to B of figure 7. The extrusion of ink from a cell which has its yolk and ink in separate vacuoles is therefore independent of the presence of food and occurs a long time before digestion of the food is completed. The curve of extrusion of ink, A, figure 8, *II*, shows now only one maximum and this corresponds to the second maximum of curve A, figure 7, and is practically identical with curve B of complete digestion. This shows then that whenever ink is included with food in the same vacuole it is retained until its accompanying

food is completely digested, because the maxima of the curves *A* and *B* of *II*, and of *B*, *I*, occur at the same time. We therefore have a demonstration of the selective extrusion among vacuoles as well as of a process of selection in feeding in *Bursaria*. Similar curves may be worked out for Sudan III or powdered aluminium.

#### SUMMARY

1. *Bursaria* has three ways of rejecting solid particles, as shown by the paths over which the particles are passed. These are: (a) the path of total rejection, shown by particles which never enter the oral apparatus; (b) the path of rejection of large particles; this being a retracing in the opposite direction of the path by which they entered; (c) the path of rejection of small particles, which leave the oral pouch by way of the base of the oral sinus and are passed backward over the ventral side of the body (fig. 1).

2. No definite path is followed by the food vacuoles during digestion, and in their passage through the cytoplasm. Residues are eliminated from a small area on the mid-dorsal side of the cell.

3. Grains of fresh hard boiled yolk of hens egg, when prepared as described (page 8), furnishes a good unit of measure of the food taken, and an easy means for determining the factors which come into play in the process of feeding in *Bursaria*.

4. The amount of food eaten and the rate at which it is eaten depends upon the physiological state of the cell (defined on page 10). This is shown to be true for fresh and for fat-free yolk, and also for indigestible substances such as aluminium, Sudan III, chinese ink, etc.

5. Change in the physiological state of the cell is indicated by the change in the total amount eaten and the rate of feeding, under the same conditions.

6. The rate of feeding is not affected in proportion to the concentration of the yolk suspension.

7. Mechanical stimulation decreases the rate of feeding or inhibits it, roughly in proportion to the degree of stimulation.

8. Rise in temperature increases the rate of feeding on yolk.

9. Continuous action of white light of high intensity had no detectable effect upon feeding on yolk.

10. Feeding may continue during stimulation by a direct electric current of sufficient intensity to control the direction of movement of the organism.

11. Bursaria can discriminate between and select non-toxic grains of yolk from among toxic ones. Whether or not Bursaria will eat yolk grains that have adsorbed a soluble substance depends upon (a) the steepness of the effective concentration gradient of the dye, between the grain and the non-toxic medium; and this in turn depends upon the amount of dye adsorbed which is subject to a reversible adsorption; (b) the specific chemical properties ('taste?') of the substance adsorbed.

12. There are strong reasons for believing that different parts of the cell are affected unequally by certain toxic substances, and that these may have a specific action upon the selection mechanism, causing a more definite rejecting reaction.

13. Yolk which has adsorbed a substance which is insoluble in water (Sudan III) is eaten as readily as fresh unstained yolk.

14. Bursaria has the power of selective extrusion among vacuoles each containing different substances eaten at the same time; vacuoles containing indigestible substances are soon extruded, while those containing food are retained. If fat-free yolk is present in the same vacuole along with the indigestible substance, then the latter is retained until digestion of the enclosed yolk has run its usual course.

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## THE ODD CHROMOSOME IN THE SPERMATOGENESIS OF THE DOMESTIC CHICKEN<sup>1</sup>

ALICE M. BORING AND RAYMOND PEARL

SIX PLATES

The spermatogenesis of several birds has been described by Guyer; the pigeon ('00), the domestic guinea ('09 a), and the domestic chicken ('09 b). The chickens used were of the Black Langshan breed. Guyer reports for this form an *X*-chromosome, which goes into one half of the secondary spermatocytes, thus giving rise to two kinds of spermatozoa, half with *X* and half without. If this *X*-chromosome is assumed to be a sex chromosome, as in so many forms, the male bird, according to Guyer's account, must be heterozygous in regard to sex.

The chicken is one of the few animals on which there have been both breeding experiments and cytological work bearing upon this point. Breeding experiments with the domestic fowl have furnished a great mass of the clearest kind of evidence regarding the inheritance of sex. On account of the importance of the point at issue, and in order that the cumulative weight of the body of independent experimental evidence may be more readily appreciated, it will be well to review briefly the literature regarding sex-linked inheritance in poultry and in other birds. We may begin this review with the case of the inheritance of the barred color pattern of the Barred Plymouth Rock which has been thoroughly studied by a number of workers. Following the papers of Spillman ('09 a and b) suggesting, on the basis of observations of crosses of Black Langshans and Barred Plymouth Rocks, that the female fowl was heterozygous, and the

<sup>1</sup> Papers from the Biological Laboratory of the Maine Agricultural Experiment Station, No. 46.

male homozygous, for sex and the barred pattern factor, this was proved experimentally to be the case by Goodale ('09) who made crosses involving the following breeds; White Plymouth Rocks (in which barring is cryptomeric), Rhode Island Reds, and Buff Plymouth Rocks. These results were confirmed and extended in a series of papers from this laboratory by Pearl and Surface ('10 a, b, c) in which Barred Rocks and Cornish Indian Games were the breeds crossed. Hadley ('10) reported the same results with the same two breeds from earlier records obtained by Cushman. Morgan and Goodale ('12) have reported in detail the results of crossing Barred Rocks with Black Langshans (the breed used by Guyer in his cytological study) and American Dominiques with Langshans. Their findings are in entire accord with those of other workers. Davenport ('06) has reported a cross between White Cochin and Tosa, in which the results are clearly to be interpreted in the same way. Recently Hadley ('13) has reported White Leghorn  $\times$  Black Hamburg crosses, which again show that the female is heterozygous and the male homozygous for sex and barring (here carried as a cryptomere by the White Leghorn). Finally, it may be said before leaving the question of the barred pattern, that in unpublished work from this laboratory it has been shown that crosses of Barred Plymouth Rocks with (a) Campines, (b) Golden Pencilled Hamburgs, (c) Black Hamburgs, (d) White Wyandottes, and (e) White Plymouth Rocks, give results in every essential accordant with those already published regarding the inheritance of this color pattern.

Turning to other characters of poultry, breeding experiments have demonstrated sex-linked inheritance of the same sort as is shown by the barred patterns, in a fairly wide range of cases. Bateson and Punnett ('11) in crosses of Brown Leghorns (and several other breeds) with Silkies demonstrate sex-linkage of an inhibiting factor influencing the mesodermal pigmentation of the Silky. Goodale ('10) shows that the Brown Leghorn color pattern is sex-linked (crosses of Brown Leghorns with White Plymouth Rocks and White Langshans). Hagedoorn ('09) shows the same essential facts regarding 'Bankiva' and 'Brown-red'

color patterns in crosses of game bantams. Davenport ('11 and '12) reports the same sort of results for color pattern inheritance in Dark Brahma  $\times$  Brown Leghorn crosses. Sturtevant ('11 and '12) in crosses of Brown Leghorn and Columbian Wyandottes finds the female heterozygous for sex and certain sex-linked patterns and color factors. One of the present writers (Pearl '12) has shown that the factor on which high fecundity depends is sex-linked in the same manner as the barred color pattern (Barred Plymouth Rock and Cornish Indian Game breeds).

All of these cases, which cover a wide range of the different known types of domesticated Gallus, and include a considerable number of different hereditary characters, agree in showing that the female fowl is heterozygous for sex and sex-linked factors, while the male is homozygous. But Guyer's (loc. cit) interpretation of his cytological observations is flatly opposed to this body of clear-cut and definite evidence, based literally on thousands of cases and undergoing continued confirmation in several laboratories in the course of routine breeding operations primarily carried on for other purposes. As has been seen a number of independent investigations have checked, repeated and extended the experimental work with fowls, always with accordant and mutually confirmatory results.

Nor is this all. There is a considerable body of definite experimental evidence indicating that in other birds than Gallus the female is the heterozygous and the male the homozygous form. Here we have the investigations of Durham and Marryat ('08) with canaries, those of Staples-Brown ('12), Cole ('12) and Strong<sup>2</sup> ('12 a and b) with pigeons, and those of Goodale ('11) with ducks.

<sup>2</sup> Strong's results are included here for the following reasons: (a) his experimental facts appear to be in no essential particular different from those of other students of sex-linked inheritance in the pigeon; (b) a simple and direct interpretation of his results, in line with other cases of sex-linked inheritance, has been given by Bridges ('13); (c) because we are entirely unable to see the logical force of Strong's own interpretation, which makes the male heterozygous. Specifically it seems to violate that fundamental logical principle, which, as most adequately stated by Sir William Hamilton, runs: "neither more, nor more onerous, causes are to be assumed, than are necessary to account for the phenomena."

The history of the present investigation is as follows: In 1907-08 there was begun in this laboratory by Dr. F. M. Surface and one of the present writers (R. P.) a preliminary study of the spermatogenesis of the domestic fowl. The inherent difficulties of the material were at once apparent. It seemed clear that with any sort of technique then available it would be literally impossible to make out a definite and connected account of the spermatogenesis, in which the objective evidence on essential points would be unequivocal and indubitable. Later the other of the present writers (A. M. B.) took up the work. A great deal of time was spent in the development and experimental extension of technique in an attempt to overcome the inherent difficulty of the material. In 1910-11 the late Dr. N. M. Stevens was interested in the matter, and from then on till the time of her death in 1912 she devoted a very considerable amount of work to this problem, partly with material (both living and preserved) supplied from this laboratory and partly with cockerels of other breeds. After her death her notes, drawings and preparations were turned over to us. As will presently appear, Miss Stevens was totally unable to confirm Guyer's account in regard to any essential point. At the Cleveland meeting (1913) of the American Society of Zoölogists, Professor Guyer announced a demonstration of his chicken preparations showing the X-element. To one of us was given a very full account of the exact technique he had used. It may be said at once that every objective fact described by Guyer in his paper regarding the so-called X-element<sup>3</sup> was clearly and successfully demonstrated. There can be no question regarding the existence of bodies which Guyer calls X-chromosomes. It is the chief object of this paper, however, to show that there is at present no valid evidence that any element which may justly be interpreted as an X-chromosome exists in the chicken.

<sup>3</sup> No preparations were at hand for demonstrating the chromosome counts in the different stages of spermatogenesis, so this phase of the matter was not gone into.

## MATERIAL AND METHODS

The material used in this investigation came from twelve pure Barred Plymouth Rock and crossbred males, raised on the poultry plant of the Maine Agricultural Experiment Station. The birds varied in age from five months to two years. Those younger than five months had no dividing germ cells. Beyond that time age seemed to have nothing to do with the number of dividing cells present, or the clearness of the mitotic figures. The testes of birds killed in the active breeding period, January to April, may perhaps contain more dividing cells, but no statistics were taken on this point, and those killed in October, November and December contained many such.

Three general methods of technique have been used in the preparations: stained sections, smears, and aceto-carmin mounts. The latter two give much better results than the first. The difficulties with much vertebrate germ cell material, as compared with insects, have been many times described, and the bird material is no exception. The chromosomes act as though they were of a sticky nature, and do not separate far apart even in prophase. Smears and aceto-carmin preparations both give a possibility of flattening out the cells, and for this reason give greater separation of chromosomes than any of the sections, no matter in what they are fixed. The material for stained sections was fixed mostly in Gilson's, Flemming's or Hermann's solutions. A testis of one bird was found in the laboratory fixed in Zenker-formalin; this was sectioned, but showed rather poor fixation. All possible schemes were tried to keep the cells in as normal condition as possible. The effort was made to transfer small pieces of the testes to the Gilson or Flemming solutions while the testis was still at the body temperature of the bird. For one bird, the Flemming and the Hermann solutions were heated to 38°C. when the pieces of testis were added in order to keep them at the normal temperature of the body of the bird until fixed. For two other birds, the whole testis hot from the bird's body, was immersed immediately in Flemming at 38°C., and cut into thin slices while in the hot solution so as to avoid any change



of temperature. But none of these precautions gained any better separation of the chromosomes. The Gilson's solution gave clearer fixation than either cold or hot Flemming or Hermann.

Very small pieces of the testes of some of these same birds were put into aceto-carmin and allowed to stay indefinitely. When examined later and spread one layer of cells thick under a coverglass, the chromosomes were sharper and clearer than in any of the sections of the material so carefully fixed. If 45 per cent acetic acid, saturated with carmin, gives good results, it seemed possible that other closely related organic acids or other concentrations of acetic acid might give even better chromosomal pictures. Consequently 45 per cent formic acid, 45 per cent butyric acid and 40 per cent chloracetic acid were tried. The butyric acid is too heavy and does not penetrate the cells at all. The formic acid fixed the cells well, but did not carry the stain in as well as the acetic. Of the different percentage solutions of the acetic acid tried, the 5 per cent did not either fix or stain well, the 100 per cent gave good sharp fixation, but would not dissolve enough carmin to stain properly. So nothing was found to improve on the 45 per cent aceto-carmin. Figures 86-91 are some of the aceto-carmin preparations.

Finally smears were made exactly as Guyer made his for the Langshan cocks. The testes were taken from an old bird in its second year. The smears were fixed in Bouin's solution and stained in iron hematoxylin. The black and white of the iron hematoxylin preparations is, especially favorable for both drawing and photographing, and the smears are much better than the sections stained in iron hematoxylin. So figures 1 to 85 are all made from smear preparations.

#### OBSERVATIONS

This paper does not attempt to work out a continuous detailed history of spermatogenesis in the Barred Plymouth Rock. A glance at the photographs and drawings on plates 1 to 6 will show that such a history would require a large amount of imagination. These plates show, however, that in some of the dividing cells, a chromatin mass appears separate from the rest

of the chromatin on the spindle, and that this, if considered by itself apart from all other elements, resembles an X-chromosome. For instance figures 11 a and 51, 22 a and 73, show equatorial plates with a separate chromosome at one side; figures 14 and 61, 23 b and 82 show metaphase spindles with a chromosome toward one end of the spindle, apparently passing to one end without dividing. Figures 34, 65 and 83 show anaphases with projecting chromatin arms at only one pole. These three appearances are usually taken as signs that an X-chromosome is present. The more conclusive and unequivocal evidence based on the number of chromosomes in the spermatogonia, primary, and secondary spermatocytes respectively is not here available, because it is impossible to count chromosomes accurately in this material.

However, to follow out the only kind of evidence available, it is clear that half the above sample figures represent primary spermatocytes, and half secondary. Now the typical X-chromosome fails to divide in only one spermatocyte division: either the first, as in Coleoptera (Stevens '06) and Hemiptera homoptera (Boring '07) or in the second, as in some Hemiptera heteroptera (Wilson '05), and Orthoptera (McClung '00). It behaves like any other chromosome in the equatorial maturation division, whichever that may be. But the apparent X-chromosome in the Barred Plymouth Rock can be seen passing undivided to one pole in both primary (fig. 65) and secondary (fig. 83) spermatocytes. This would result in one-quarter of the spermatozoa with X and three-quarters without it. Following this further; if the chicken were like the majority of forms with sex chromosomes so far studied, and had one less chromosome in the male than in the female, there would be on the basis of the above observations a sex ratio among chickens of one female to three males. Davenport's ('06) work on poultry, as well as very extensive unpublished statistics on the point in the records of this laboratory, show that such a ratio does not exist in this form.

Another possibility is that the X-chromosome may divide in either the first or second spermatocyte division. Edwards describes a case of this in *Ascaris* ('10), but this is a rare occurrence.

That this is not the explanation in the Barred Plymouth Rock will be shown by the statistical tables given later in this paper (pp. 61-62):

It is interesting to notice that it is easy to pick out from either the photomicrographs or the drawings a series of pictures which, taken by themselves, would show an *X*-chromosome to be present and to find it dividing in the first maturation division. Figures 26 a, 30, 34, 36 c, 35 a, 35 b are such a series of photographs, and figures 45, 62, 66, 70, 78, 84 such a one of drawings. Further, figures 3 a, 17 a, 20, 22 a, 23 b, and the series 39, 54, 63, 77, 82, 83, taken by themselves, show just as conclusively that an *X*-chromosome is present and fails to divide in the second spermatocyte division. Still further, a series of figures made by a combination of the figures of the previous two series, for example, figures 3 a, 17 a, 20, 36 c, 35 a, 35 b, or 39, 54, 63, 70, 78, 84 shows that there is no *X*-chromosome present at all.

The above *reductio ad absurdum*, proving three conflicting hypotheses out of the same material, is the crux of the argument of this paper. In difficult cytological material, such as this, one can prove anything one wants, by picking out certain cells and ignoring all others. The only way to find out the real facts is to study a large number of cells and find out what happens in the majority. So this investigation comes to a focus around the question: Is this so-called *X*-chromosome in the Barred Plymouth Rock present in enough cells to be justly regarded as the homologue of the *X*-chromosome in insect spermatogenesis?

The following statistics were worked out to answer this question. Slides were systematically examined by means of the mechanical stage, and every clear dividing cell within a certain area was recorded under one of the twelve headings in table 1. Several nuclei are often grouped in one individual mass of cytoplasm, as described by Guyer for the Langshan birds ('09). It is easy to distinguish the second spermatocytes from the first by the amount of chromatin and the size of the spindle. The criteria used for the presence of *X* were those used by Guyer for the Langshan material, either a separate mass of chromatin as in figures 10 and 14, or a very conspicuous arm protruding

from the mass, as in figures 1 and 13. On this basis, 1003 cells were grouped into six classes with *X* and six classes without *X*. The six classes are (1) first spermatocyte, equatorial plate; (2) first spermatocyte, metaphase spindle; (3) first spermatocyte, anaphase; (4) second spermatocyte, equatorial plate; (5) second spermatocyte, metaphase spindle; and (6) second spermatocyte, anaphase. The results are given in table 1. These figures plainly show that the so-called *X*-chromosome is an exception rather than a rule. Eighty-one cells with *X* out of 1003, that is 8.07 per cent, is obviously too few to justify a generalization as to the specific character of the element, even with all the possible chances of its escaping notice. This table also gives numerical expression to the difficulty before mentioned, that *X* appears in

TABLE 1

CELLS OF TESTIS OF BARRED PLYMOUTH ROCK	I SPC. EQ. PL.	I SPC. META.	I SPC. ANA.	II SPC. EQ. PL.	II SPC. META.	II SPC. ANA.	TOTAL I SPC.	TOTAL II SPC.	TOTAL
No. of cells	267	245	63	120	248	60	575	428	1003
No. of cells with <i>X</i>	40	23	5	6	5	2	68	13	81
No. of cells without <i>X</i>	227	222	58	114	243	58	507	415	922
Per cent with <i>X</i>	14.97	9.38	7.93	5.00	2.01	3.33	11.82	3.06	8.07

both first and second spermatocyte divisions. If this were a form where *X* passed undivided to one pole in the first spermatocyte division, 11.82 per cent cells showing it in the first spermatocyte would be too few, and 3.06 per cent in the second spermatocyte would be too many. If on the other hand this were a case where the odd chromosome failed to divide in the second spermatocyte division, 11.82 per cent cells showing it in the first spermatocyte division would be too many, and 3.06 per cent too few. So the actual facts will fit neither case. And we see now why the situation found by Edwards in *Ascaris* where the *X*-chromosome divides sometimes in the first spermatocyte division and sometimes in the second, will not answer here: 8.07 per cent is still not enough cells with *X*, even allowing for this possibility of variation in behavior.

In the last paragraph it was assumed that *X* would not escape notice in many of the cells of a form where present, that is, that the element would be visible if present, in approximately 50 per cent of all dividing spermatocyte cells, 100 per cent of first and 0.00 per cent of second spermatocyte cells, or vice versa, 100 per cent of second and 0.00 per cent of first. But any one who has worked much in the cytology of germ cells knows that there are good reasons why *X* sometimes escapes notice when present. It does not by any means always take up an eccentric position in the equatorial plate; it does not always show itself in the side view of the metaphase spindle by projecting at one side or by appearing visibly univalent instead of bivalent; neither does it

TABLE 2

CELLS OF TESTIS OF PHILAEUS SPUMARIUS	I SPC. EQ. PL.	I SPC. META.	I SPC. ANA.	II SPC. EQ. PL.	II SPC. META.	II SPC. ANA.	TOTAL I SPC.	TOTAL II SPC.	TOTAL
No. of cells.....	22	128	423	34	69	324	573	427	1000
No. of cells with <i>X</i> ..	14	111	369	0	0	0	494	0	494
No. of cells without <i>X</i>	8	17	54	34	69	324	79	427	506
Per cent with <i>X</i> .....	63.63	88.43	87.23	0	0	0	86.21	0.00	49.4

always lag behind the dividing chromosomes during the anaphase of the mitosis. In order, then, that the conclusions of the previous paragraph might be based on facts and not on an assumption, a similar statistical study was made of 1000 dividing cells of a bug, *Philaenus spumarius*, the spermatogenesis of which has been previously worked out and found to exhibit a typical unpaired sex chromosome (Boring '13). The cells were picked out in the same way with the mechanical stage, systematically at random, all the clear ones in a certain area being catalogued. The results are seen in table 2, which shows that the previous assumptions were indeed very close to the facts. Out of 1000 cells, 49.4 per cent show *X*, none of the second spermatocytes show it as a chromosome behaving differently from the others, while in 86.21 per cent of the first spermatocytes, it can be differentiated. In the equatorial plate, there were only 8 out of 22

cases, where the *X*-chromosome was not eccentric. On the metaphase spindle, there were 17 out of 128 cells, where the bivalent character of the other chromosomes was not striking enough to make the difference visible. Among the anaphases, only 54 out of 423 cells failed to show a lagging chromosome at one pole; in these it had probably already reached its destined pole and lay in the same plane with the others. A comparison of these figures with those for the Barred Plymouth Rock are certainly convincing: the Barred Plymouth Rocks do not show enough cells with an apparent *X*-chromosome to make it even mildly probable that this is a sex chromosome like the *X*-chromosome of insects. In the Barred Plymouth Rock there are 11.82 per cent primary spermatocyte cells with a possible *X*-chromosome, as contrasted with 86.21 per cent in the insect. In the secondary spermatocytes, the Barred Rock has 3.06 per cent cells with an apparent *X*, while the insect has 0 per cent.

We have seen then that the small proportion of cells with a possible *X*-chromosome casts some doubt on its interpretation as such. We will examine next some of the individual cells where this separate mass of chromatin appears, and see whether it is always consistent in shape and size, that is, whether it can be regarded as a definite individual chromosome of any kind whatsoever.

The figures, both photomicrographs and drawings, plainly show that the shape of the separate mass of chromatin varies. It may be round, as in figures, 11 a, 12 b, 14, 16, 22 d, 27, 28 a, 28 c, 50, 77 and 82. It may be rod-shaped, as in figures 5, 8 b, 34, 49, 52, 53, 57, 65. It may be *U* or *V*-shaped, as in figures 9, 33, 46, 47, 48, 60, 61. It may be three-parted, as described by Guyer ('09) for the Langshan cock, and shown in the following figures of this paper: 10, 26 b, 43, 44, 74, 83. It may even be four-parted, as in figures 45, 62, 66 and 73. Besides these, there are many cells where rod-like arms project from the mass of chromatin, which are rated by Guyer as *X*-chromosomes (figs. 1, 13, 29, 38, 40, 55, 56, 81).

Even more striking than the variation in shape is the variation in size. This ranges all the way from a tiny speck (figs.

2 b, 2 c, 14, 49, 51) up to a piece almost half the volume of the whole chromatin mass (figs. 7 b, 8 b, 25 b, 25 c, 43, 75).

Beside the above-mentioned inconsistencies in shape and size, in some cells there is more than one separate piece of chromatin. Figures 9, 27, 48, 49, 53, 83 show two separate chromosomes, and figure 28 c shows three, while figure 75 may show four. These are cases where the two or three X-chromosomes are all distinctly separated from the chromatin plate. If one counts the projecting arms and pieces lying close to the large mass, yet apparently separate chromosomes, there are many more cases with more than one X-chromosome. Figure 1 has four arms; figure 28 a has one round X-chromosome and one arm; figure 46 has one V-shaped chromosome and one arm; figure 49 has one round odd chromosome, one rod-shaped and one arm; figure 52 has one separate rod and one rod-like arm; figure 61 has one V-shaped element and two arms. If it is legitimate to call an arm an X-chromosome when there is no separate chromosome to call X, then it ought to be equally legitimate to count an arm as an X-chromosome, when a separate chromosome is present, and to conclude that there is more than one X-chromosome in such cases.

A study of the anaphases shows that in this Barred Plymouth Rock material, a lagging condition, as indicated by a projecting arm cannot safely be regarded as indicating a real difference in behavior from other chromosomes, for there are in actual fact as many cases with corresponding lagging arms at both poles of the anaphase spindle as with one at only one end. Figures 18 b, 19, 21, 67, 68, 69 are spindles where this is the case. This evidently means merely that these Barred Plymouth Rock chromosomes do not always separate as evenly and as synchronously as those in insect material.

We have seen that the so-called X-chromosome of the Barred Plymouth Rock male germ cells varies in shape, size and number. If it is such a variable element, it is evident that many of the figures used in this paper are capable of varying interpretations. Let us run over the figures to see what possible interpretations we find for some of them. Figure 1 is a primary spermatocyte.

cyte equatorial plate. It has four projecting arms. Perhaps these are four *X*-chromosomes. But two arms are longer than the others. Perhaps only the two longer arms are *X*-chromosomes. The two long arms are rod-shaped, while the two short ones are rounded. If there is only one *X*-chromosome and that is round, it must be one of the short arms; if it is rod-shaped, it must be one of the longer. If there are two *X*-chromosomes, one might be rod-shaped and one round, or both might be round, or both might be rod-shaped. Or it might much more probably be that there is no *X*-chromosome at all, and all four arms are only ordinary bivalent chromosomes, which happen to project a little from the plate of chromosomes.

Figure 4 is a primary spermatocyte equatorial plate and may have a round *X* to the left and a rod-shaped one to the right, or either one of them may be *X*, while the other is a bivalent chromosome. Or again, there is nothing about either which stamps it even probably as an *X*-element, so there may be no *X* at all.

Figure 12 b is a first spermatocyte equatorial plate and may have either one or two *X*-chromosomes, according to whether one counts the round body which touches the mass of chromosomes as one or not. It does not happen to be quite as far away as the entirely separate one, but there is no other indication of its being different in character.

Figure 13 is a side view of a primary spermatocyte metaphase spindle, the arm to the left, pointing toward the upper pole may represent an unpaired chromosome, but on the other hand, there is no surety that this is univalent, while the rest of the mass is composed of bivalent chromosomes.

Figure 33 is a first spermatocyte metaphase spindle. The body to the right may be a *V*-shaped *X*-chromosome, or it may be a three-parted *X*-chromosome, or it may be an ordinary dumb-bell-shaped bivalent chromosome in the process of dividing.

Figure 18 b is a first spermatocyte anaphase. Any one of the three arms to the left might be a lagging *X*-chromosome, but the general scattered condition of the chromatin would make it just as reasonable to interpret all three as ordinary chromosomes. The middle one at least has a mate at the right pole. When



the microscope is focused on the right pole instead of the left, arms appear which might be considered the mates of the other two arms of the left pole.

Figure 66 is also a first spermatocyte anaphase. The three or four-parted body toward the upper pole might be an *X*-chromosome, but the chromosomes are so loosely grouped here, and the separate body is so close to the pole that such an interpretation, has no particular probability in its favor.

Figure 22 a is a second spermatocyte equatorial plate. The body to the left may be one rod-shaped *X*-chromosome, or two round ones, or not an *X*-chromosome at all.

Figure 81 is a second spermatocyte metaphase spindle. The part projecting toward the lower pole might be a univalent *X*-chromosome, or one part of a large bivalent dividing.

Figure 83 is a second spermatocyte anaphase. The three-parted body at the lower pole may be an *X*-chromosome, or it may be the mate to the projecting mass at the upper pole. The small round chromosome at the lower pole may be a second *X*-chromosome, or it may be one of the ordinary chromosomes with its mate fused in the mass at the upper pole.

Similar varying interpretations might be given for most of the figures. There would then appear to be abundant justification for the statement that there is no valid evidence of the existence of an *X*-chromosome in the Barred Rock male, on the following grounds:

a. The small percentage of cells containing any element which, on the most liberal interpretation, could be regarded even as a possible *X*-chromosome.

b. The inconstancy of the shape, size and number of these possible *X*-chromosomes.

c. The fact that many (not to say most) of them would never be considered to be sufficiently proved as *X*-chromosomes if this were insect material.

The 11.82 per cent of first spermatocytes and the 3.06 per cent of second spermatocytes classified in the table as 'with *X*,' however, demand further attention. The projecting arms and those lying in close juxtaposition to the equatorial plate have

had an explanation offered for them in the above paragraphs, but there are some cells such as figures 10, 14, 28 c, 53 and 61 where there is a distinctly separated piece of chromatin. It seems, however, a reasonable explanation that these are one, two, or several of the ordinary chromosomes that happen to be too far from the others to fuse into the general mass. If this material were such that one could count the number of chromosomes in the first and second spermatocytes, and see tetrads formed for division, one could tell absolutely whether the pieces were extra chromosomes or not. But the chromosomes act almost as though they were sticky on the surface, and those that happen to touch each other stick close together and stain as though fused. Comparing figure 53 with 38 it is clear that the right hand piece is no further from the center of the mass in the first than in the second case, only it is one of the smaller chromosomes and therefore did not touch the mass and stained separately. If one compares figures 52 and 40, the same thing is seen: the rod is a little shorter and does not quite touch the mass in 52, while it just touches it in 40.

A study of all the photographs of the primary spermatocyte equatorial plates as shown on plate 1 and all the drawings of the same stage, as shown on plate 4, in the order in which they are arranged on the page will show a gradual transition from the large mass with projecting arms, through the smaller mass with separate chromosomes nearby or even touching, to the small mass with chromosomes quite far away. A similar study of the series of photographs and drawings of the side views of the metaphase spindles of the first spermatocytes, as shown on plates 2 and 5, indicates the same sort of transition.

The difference in size of these chromosomes left outside of the general mass in different cells, would indicate a regular and constant size difference among the chromosomes. Some of the clearest aceto-carmin preparations give additional evidence for this (figs. 87, 89, 90, 91). In these preparations it was more clearly possible to see all the separate chromosomes than in preparations by any other technique. But even here counting proved illusory. Figure 86 shows six chromosomes, figure 87 seven,

figure 88 eight, and figures 89, 90 and 91 nine. Figures 50 and 85 are the only two cells from smear preparations, where counting was at all possible, and here it seems rash. Both of these cells seem to show nine chromosomes. In the notes and rough sketches left by Dr. N. M. Stevens on the Barred Plymouth Rock material she has recorded more cells where she could count nine than any other number, but she had counts recorded all the way from 5 to 22.

Another note of Dr. Stevens indicated that she did not find any sign of the double reduction recorded by Guyer for pigeons, chickens and guineas. Figures 70 to 75 look as though the number of chromosomes present in the secondary spermatocytes must be more than half of nine, and figure 90, an aceto-carmin preparation of a secondary spermatocyte, shows nine clearly as the second spermatocyte number. However, these facts as to number are too meager to be offered as anything more than isolated observations. The result of this investigation is to show that there is no good evidence for a sex chromosome in the germ cells of the Barred Plymouth Rock males and that therefore there is no cytological evidence to conflict with the experimental evidence that the female fowl is heterozygous and the male homozygous for sex.

#### SUMMARY

There is no good observational or statistical evidence of the existence of a sex chromosome in males of domestic chickens of the Barred Plymouth Rock breed. In 11.82 per cent of first spermatocytes and 3.06 per cent of second spermatocytes, there is a piece of chromatin similar to that called an X-chromosome by Guyer in Langshan males. This is not to be regarded as an X-chromosome in Barred Plymouth Rock males, because:

1. It is present in spermatocytes of both orders.
2. A statistical study of Barred Plymouth Rock cells in comparison with those of the hemipteran *Philaenus spumarius* shows that it is present in too few I spermatocytes and in too many II spermatocytes, or vice versa.

3. It is still present in too few cells, if it should be interpreted as one of those rare cases where the odd chromosome divides in either the I or II spermatocyte division.

4. It varies in shape.

5. It varies in size.

6. It varies in number.

7. In no single cell is it of such a shape or size, or in such a position, that it could not readily be interpreted as anything else than an X-chromosome.

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## EXPLANATION OF FIGURES

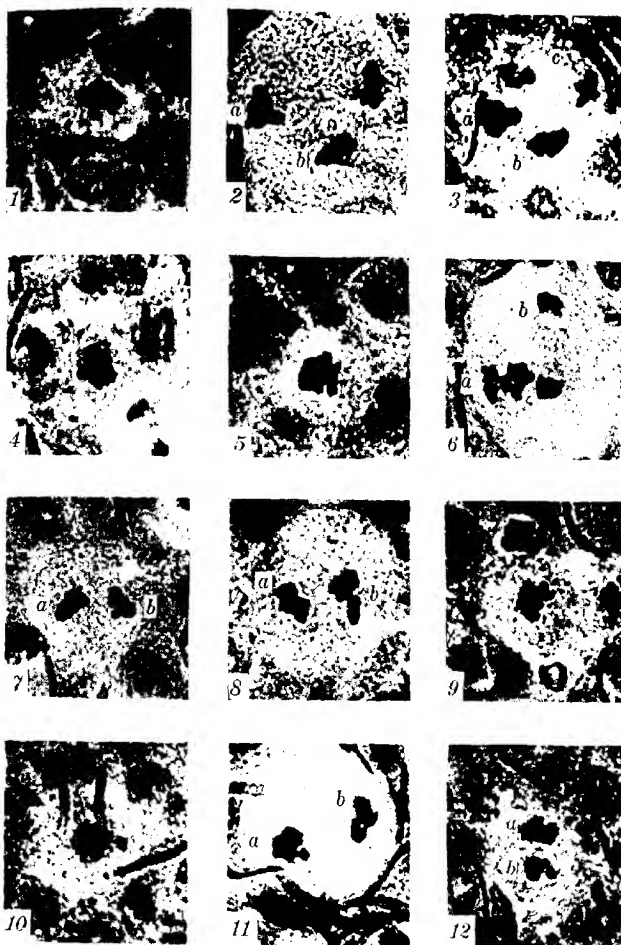
The photomicrographs of plates 1 and 2 were made with a Leitz  $\frac{1}{2}$  oil immersion and 6 ocular, and enlarged in the taking so that they give a magnification of 1150. Those of plate 3 were made with a Zeiss 2 mm. objective and 6 ocular, giving a magnification of 750. The drawings of plates 4 to 6 were made with a camera lucida, at the level of the table, and with a  $\frac{1}{2}$  oil immersion, and 12 compensating ocular, no reduction. Figures 1 to 85 inclusive, both photographs and drawings are from smear preparations fixed in Bouin and stained in iron hematoxylin. Figures 86 to 91 are of aceto-carmin preparations. The photographs were made by Mr. Royden L. Hammond.

## PLATE 1

### EXPLANATION OF FIGURES

#### *First spermatocyte division stages*

- 1 Equatorial plate with four projecting arms.
- 2 a Equatorial plate with one projecting arm.
- 2 b and 2 c Metaphase spindles, each with a very small separate chromosome.
- 3 a Equatorial plate, chromosomes closely massed together.
- 3 b Metaphase spindle with no *X*-chromosome.
- 3 c Anaphase with projecting arm at the left pole.
- 4 Equatorial plate with a round chromosome to the left and a rod to the right.
- 5 Equatorial plate with a rod to the right.
- 6 a Equatorial plate, chromosomes much scattered.
- 6 b Anaphase with no possible *X*-chromosome.
- 7 a Equatorial plate, chromosome is fused.
- 7 b Equatorial plate, chromosomes in two nearly equal masses.
- 8 a Metaphase spindle with no possible *X*-chromosome.
- 8 b Equatorial plate, large separate rod, one-third the size of whole chromosome mass.
- 9 Equatorial plate, V-shaped chromosome to right.
- 10 Equatorial plate, two separate chromosomes, one round to left, and one three-parted to right.
- 11 a Equatorial plate, one round chromosome to the right.
- 11 b Equatorial plate, separate round mass, one-third the size of the whole chromosome mass.
- 12 a Equatorial plate, no possible *X*-chromosome.
- 12 b Equatorial plate, two separate round chromosomes to the right.





## PLATE 2

### EXPLANATION OF FIGURES

#### *13 to 18 a First spermatocyte metaphase spindles*

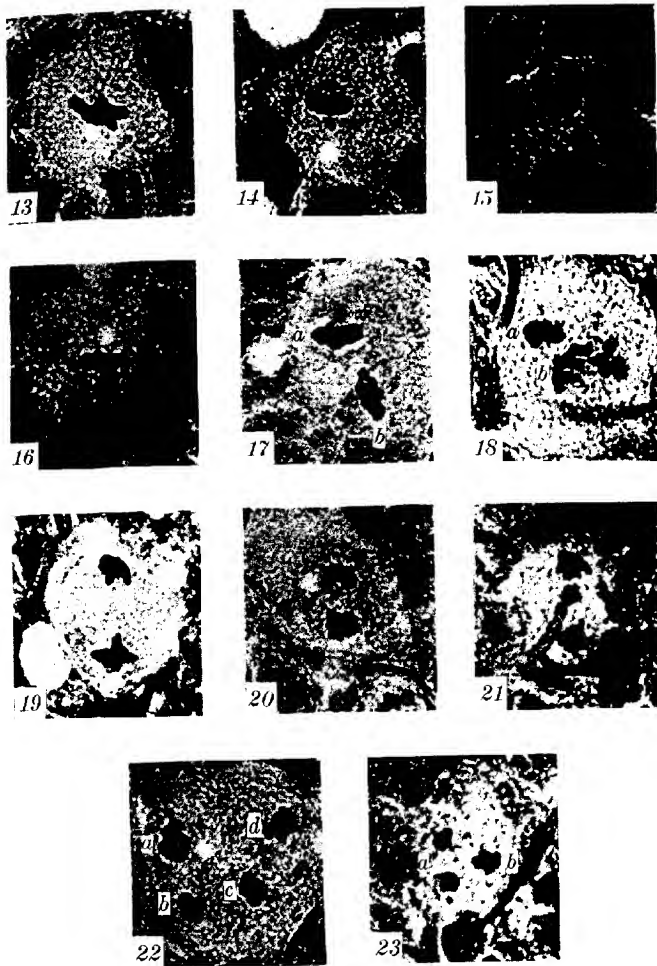
- 13 Extra arm to left toward upper pole.
- 14 Small round separate chromosome toward lower pole.
- 15 and 16 Round chromosome projecting from the mass toward the lower pole.
- 17 a and b No possible X-chromosome present.
- 18 a No possible X-chromosome present.

#### *18 b to 21 First spermatocyte anaphases*

- 18 b Left pole of spindle more clearly in focus than right; at least one of three projecting arms at the left has a corresponding arm at the right.
- 19 Two projecting arms at upper pole, and one at the lower.
- 20 No possible X-chromosome.
- 21 Projecting arm at right of both upper and lower poles.

#### *22 and 23 Second spermatocytes*

- 22 a Equatorial plate, rod to left.
- 22 b Metaphase spindle, small round projection toward lower pole.
- 22 c Equatorial plate, no possible X-chromosome.
- 22 d Equatorial plate, separate round chromosome to left.
- 23 a Anaphase, no possible X-chromosome.
- 23 b Metaphase spindle, small round projection toward lower pole.



### PLATE 3

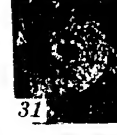
#### EXPLANATION OF FIGURES

##### *24 to 34 First spermatocyte divisions*

- 24 a Metaphase spindle, projecting arm toward left pole.
- 24 b Equatorial plate, projecting arms.
- 25 a Equatorial plate, no possible X-chromosome.
- 25 b and c Equatorial plates, mass of chromosomes in two pieces.
- 26 a Equatorial plate, round chromosome to right.
- 26 b Metaphase spindle, three-parted chromosome toward upper pole.
- 27 Equatorial plate, two separate round chromosomes.
- 28 a Metaphase spindle with round chromosome to the right, and an arm to the left.
- 28 b Metaphase spindle, one small round chromosome to left.
- 28 c Equatorial plate, three separate round chromosomes.
- 29 Metaphase spindle, corresponding projecting arms to the right toward each pole.
- 30 and 31 Metaphase spindle, one round chromosome toward lower pole.
- 32 Metaphase spindle, projecting arm toward upper pole, and round chromosome toward lower.
- 33 Metaphase spindle, V-shaped chromosome to right.
- 34 Anaphase, projecting rod-like arm only at lower pole.

##### *35 to 37 Second spermatocyte divisions*

- 35 a Metaphase spindle, no possible X-chromosome.
- 35 b Anaphase, no possible X-chromosome.
- 36 a and d Equatorial plates, out of focus.
- 36 c and e Equatorial plates, no possible X-chromosome.
- 36 b Anaphase, no possible X-chromosome.
- 37 a and c Equatorial plates, out of focus.
- 37 b Metaphase spindle, no possible X-chromosome.
- 37 d Equatorial plate, round chromosome toward upper side.



## PLATE 4

### EXPLANATION OF FIGURES

#### *38 to 53 First spermatocyte equatorial plates*

- 38 Chromosomes massed with projecting arms.
- 39 Chromosomes loosely massed with space in center.
- 40 Chromosomes scattered, but still all connected.
- 41 Chromosomes massed, with a V-shaped body partially separated to the right.
- 42 Chromosomes massed, with a three-parted body partially separated to the right.
- 43 Three-parted separate body, one-third the size of the whole chromosome.
- 44 Three-parted separate body, small in proportion to size of whole chromosome plate.
- 45 Four-parted separate chromatin body to the right.
- 46 V-shaped separate chromatin body to right.
- 47 V-shaped separate chromatin body at lower side.
- 48 Two separate chromosomes, one round and one V-shaped.
- 49 Two separate chromosomes, one small round one and one rod-shaped.
- 50 One round chromosome somewhat separated from the rest. The number of chromosomes here might be counted as nine.
- 51 One small round separate chromosome to the right.
- 52 Two rod shaped chromosomes, one separate and one attached.
- 53 Similar to 52, but rods shorter.



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## PLATE 5

### EXPLANATION OF FIGURES

#### *54 to 62 First spermatocyte metaphase spindles*

- 54 No possible *X* present.
- 55 and 56 Two projecting arms.
- 57 One projecting rod toward lower pole.
- 58 and 59 Projecting round chromosome toward lower pole.
- 60 *V*-shaped chromosome toward lower pole.
- 61 *V*-shaped chromosome toward lower pole and two projecting arms toward upper pole.
- 62 Four-parted body toward lower pole.

#### ● *63 to 69 First spermatocyte anaphases*

- 63 No possible *X*-chromosome present.
- 64 Projecting arm at upper pole.
- 65 Projecting rod-like arm at lower pole.
- 66 Separate four-parted body at upper pole.
- 67 to 69 Corresponding projecting arms at both poles.



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62



63



64



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66



67



68



69



## PLATE 6

### EXPLANATION OF FIGURES

#### *70 to 84 Second spermatocyte divisions*

70 to 72 Equatorial plates, no separated chromosome. Apparently more than one-half as many chromosomes as in I spermatocyte, although counting is impossible.

73 to 77 Equatorial plates, with separated chromosomes, varying in number and shape.

78 and 79 Metaphase spindles, no possible X-chromosome.

80 and 81 Metaphase spindles, with projecting arms toward lower pole.

82 Metaphase spindle, one round separate chromosome toward lower pole.

83 Anaphase, two separate chromosomes toward lower plate, one round and one three-parted.

84 Anaphase, no X-chromosome present.

85 First spermatocyte equatorial plate. One of the few cells in the smear preparations where it is possible to try to count chromosomes, the number might be nine.

#### *86 to 91 Aceto-carminé preparations*

86 First spermatocyte equatorial plate, where there are apparently 6 chromosomes.

87 First spermatocyte equatorial plate, where there are apparently 7 chromosomes.

88 First spermatocyte equatorial plate, where there are apparently 8 chromosomes.

89 First spermatocyte equatorial plate, where there are apparently 9 chromosomes.

90 Second spermatocyte equatorial plate, where there are apparently 9 chromosomes.

91 First spermatocyte anaphase, where there are apparently 9 chromosomes at each pole.



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# THE EFFECT OF RADIUM RADIATIONS ON THE FERTILIZATION OF NEREIS

CHARLES PACKARD

THREE PLATES

## CONTENTS

Introduction.....	85
The nature of the radium radiations.....	86
Historical review.....	86
Methods.....	91
The normal development of Nereis.....	92
Experimental.....	93
a. The fertilization of normal eggs by radiated spermatozoa.....	93
b. The development of eggs radiated before fertilization.....	98
c. The development of eggs radiated after fertilization.....	100
d. The development of eggs radiated before and after fertilization.....	106
e. The development of eggs normally fertilized, and exposed to radium.....	107
f. Subsequent development of the embryo.....	109
g. Development of eggs radiated before and after fertilization with normal sperm.....	109
Discussion.....	112
Summary and conclusion.....	119
Literature cited.....	121

## INTRODUCTION

The radiations of radium have been used as a stimulus to protoplasmic activities from the time they were first discovered. The early experiments were made with little knowledge of the nature of the stimulus and with none whatever as to its probable effect on living matter. It was found that the rays affected the tissues in very different ways, acting as a stimulus to growth in some tissues, and as an inhibitor in others. Thus, seedlings have, in some instances, been accelerated in growth, and the action of certain enzymes (pepsin, diastase, etc.) favored by exposure to the rays. On the other hand, an exposure to radium produces a marked retardation in the growth of certain tissues and a characteristic degeneration in some of the cell constituents. The latter effects

are found chiefly in the rapidly growing structures, such as embryonic or regenerating tissue. The nature of the injury has been studied particularly in the former type, and the generalizations made on the effect of radium radiations have been based chiefly on such investigations. A study of the cytological details of the changes thus brought about has been the point at issue in the most recent studies on this subject and is the object of the present investigation.

#### THE NATURE OF THE RADIUM RADIATIONS

A discussion of the nature of the radium radiations need not be entered into fully in this paper. It may be mentioned simply that the three types of rays apparently produce rather different effects. The alpha rays, which form the greatest part of the energy given off, are wholly shut out by the glass or mica screens which are usually arranged for the protection of the salt. As the apparatus used in the following experiments and in the investigations of all those who used living tissues as an object for study effectually screened the alpha rays, they may be disregarded in this discussion. The beta and gamma rays are able to penetrate thin glass, but their activity is greatly diminished in the transit. Their effect on protoplasmic activities is, in general, an injurious one, although Congdon ('12) found some evidence of acceleration in the rate of development in the eggs of *Drosophila* due to the secondary gamma rays. In this case only the eggs which received a very weak stimulus were accelerated. The injurious effects found by practically all observers will be discussed in the next section.

#### HISTORICAL REVIEW

It has been found that different tissues show a great diversity in the nature of their response to the radiations of radium. Thus, the spermatogonia and the spermatocytes in the testis of the rat may be killed or greatly injured while the surrounding Sertoli cells and the connective tissue show no effects whatever. The same is true for ova and the follicle cells. Körnicke ('05) who exposed to radium the growing root tips of *Lilium* for periods of

one hour to three days, found that the most noticeable effect was on the nucleus of the cells, the protoplasm apparently undergoing no change. The chromatin was clumped together, and in some cases formed a homogeneous ball. He also found in the tetrad cells a great number of small nuclei, and many extra-nuclear nucleoli.

Guilleminot ('08) found similar changes. With intense radiation (200 mg. of the pure bromide) the nucleus of the pollen grain lost all power of division after the fertilization of the ovule. With weaker stimulus the resulting embryo was small and abnormal in various respects. If the stimulus is applied during fertilization the injurious effect appears early in development, and the plant is unable to repair the injury. If the adult tissue of the plant is exposed, very little effect can be noted.

Experiments on the effect of radiations on the germ cells and on the fertilized egg have been carried on by numerous observers. The first of these was Bohn ('03) who used the frog and sea-urchin as the basis of investigations. In general he found that the radiations produce a retarding and injurious effect on the early development of both of these forms. On exposing sea-urchin sperm he found that they very soon lost their motility and failed to fertilize the eggs, a fact not borne out by the experiments of G. Hertwig in the case of *Parechinus miliaris*, or by my own on *Arbacia punctulata*.

Perthes ('04) has described similar effects of radiation on the fertilized eggs of *Ascaris megalocephala*. Schaper ('04) and Levy ('06) have described the abnormal larvae arising from the radiated, fertilized frog's egg. The abnormalities consisted in the peculiar shape of the larvae, and the pathological condition of the nervous system. The vascular system also was much injured. Schaper believed that the abnormalities in the embryo were due in a large measure to the destructive action of radium radiations on the yolk, the lecithin being destroyed through the ionizing effect of the radiations. This theory, first proposed by Schwarz ('03), will be discussed later.

Bardeen ('11) has found that X rays (which are similar to the gamma rays of radium) produce a marked effect on both eggs and sperm cells of frogs so that they give rise to abnormalities. The

radiation of the egg produces a greater injurious effect than that of the sperm. In early cleavage susceptibility of the cells is increased, but in later stages he found, in common with many other observers, that the susceptibility is greatly diminished.

A more critical examination of the effect on the embryo of radiating the germ cells has been made by O. and G. Hertwig who also used the frog as a basis of investigation. In four series of experiments they exposed first the fertilized eggs (*A* series); the sperm (*B* series); the unfertilized egg (*C* series); and both eggs and sperm before fertilization (*D* series). In general they found that the exposure of the egg or sperm alone produced smaller abnormalities than the exposure of the fertilized egg. In the *A* series and in the *D* series the development was affected in proportion to the length of exposure and the intensity of the radiation. The same is true, up to a certain point, for the *B* and *C* series. But if the sperm or the egg is exposed for a long time the resulting embryo is fairly normal. In such cases, according to O. and G. Hertwig, development is parthenogenetic, since the sperm nucleus never becomes a part of the cleavage nucleus, but serves merely to initiate development. The same is true when the egg is radiated. The sperm nucleus and not the egg nucleus is active.

The embryos developing after the various types of treatment show characteristic abnormalities. Gastrulation may be very abnormal and the older embryos much bent, as described by Schaper ('04). The internal structures also show great changes. Most seriously affected is the nervous system in which the cells are abnormal and the nuclei broken into granules. The blood system also is injured. Physiological abnormalities are as marked as the morphological. Growth is greatly retarded, due to the slow rate of cell division. Though the muscles develop they are paralyzed for lack of an adequate nervous system. The nuclear disturbances in the affected cells are similar to those found by earlier investigators, but the plasma of the cells appears to be unchanged.

A further study of the exact nature of the injury to the nucleus has been made by P. Hertwig ('11) and G. Hertwig ('12). The

former found that the fertilized egg of *Ascaris megalocephala*, after exposure to radium, shows much the same type of abnormality that had been observed by others, namely, that the chromatin alone is affected, being broken up into numerous irregular granules. In division these granules are not exactly equally divided, but part go to one blastomere and part to the other. The astral system and the plasma are not affected at all. Miss Hertwig concludes that the effect of the radium radiations is a direct one on the chromatin, and not indirect, as Schwartz and Schaper claimed.

G. Hertwig ('12) has studied the effect of fertilizing the normal sea urchin egg with radiated sperm. The sperm remains active after twelve hours of intense radiation. But such sperm, even though motile and able to penetrate the egg, is incapable, in many instances, of fusing with the egg nucleus. Inside the egg it remains as a compact mass near the latter which divides, being provided with centrosomes derived from the sperm. In some instances it may become involved as a foreign body in the spindle of the dividing egg nucleus, in which case it causes abnormalities in the distribution and form of the egg chromosomes. In division it usually goes to one blastomere, and in later divisions may operate to produce very abnormal mitoses resulting in an abnormal larva. In other instances it may fuse with the egg nucleus but even then it behaves abnormally and is eliminated during the subsequent divisions. The main point is that the embryos arising from this treatment are parthenogenetic. Thus cytological study has confirmed his earlier hypothesis based on the experiments with the frog's egg.

Since there is no visible evidence that the protoplasm of the sperm or fertilized egg has been injured, the Hertwigs conclude that only the chromatin is affected. This conclusion is supported by the fact that the harmful effect of the radiations is much greater in the fertilized egg, with twice as much chromatin, than in the normal egg fertilized by radiated sperm.

Two hypotheses have been advanced to explain the phenomena that have been described. The first, proposed by Schwarz ('04) was based on the fact that egg yolk is decomposed by exposure to the



radium radiations. Although the matter was not chemically determined, it seemed probable that the lecithin was broken up into cholin and tri-methyl-amine and other end products of lecithin decomposition. Lecithin has been found by many investigators in all cells, especially in egg yolk, spermatozoa, pollen cells, plant spores, growing buds, and in all rapidly growing tissue. If then, it is destroyed such cells must necessarily be unfavorably affected.

Against this hypothesis can be raised a number of objections. In the spermatozoön there can be very little lecithin, and any amount that is destroyed would be so small as to be nearly negligible. Yet Hertwig has stated that exposure of frog sperm for one minute is sufficient to produce some abnormality in the embryo. It is hard to imagine that this is due to the amount of lecithin decomposed in so brief a period. On the other hand, the egg contains a relatively enormous quantity, so that the effect should be correspondingly greater, yet such is not the case in the frog. The experiments of Bardeen show only a slightly greater injury when the egg is exposed. It is also found that the fertilized egg, when radiated, develops very abnormally even though the exposure is brief. These facts indicate that the lecithin hypothesis cannot explain all of the phenomena although the decomposition of the lecithin under radiation is an actual fact.

The second hypothesis, advanced by O. Hertwig is called by its propounder a 'Biological hypothesis' although fundamentally chemical in its nature. It is assumed that the radium radiations affect only the chromatin and that all the abnormalities that result from radiations arise in consequence to the injury to that substance. The chromatin, under the influence of the radiations produces a 'contagium vivo' which acts like a living ferment in that it increases at each cell division. Thus an originally small amount generated in the sperm will make itself felt after many cleavages, while a large amount generated in the fertilized egg will produce abnormalities almost at once. In cases in which the spermatozoa have been radiated for a long time it is assumed that the 'contagium vivo' has increased to such an extent that it has killed itself and at the same time affected the dividing power of

the chromatin. The destruction of the poison by prolonged radiation makes it possible for the egg to develop parthenogenetically. When the fertilized egg is radiated enough poison is generated to prevent cell division entirely.

There is no doubt that some substance is stimulated to activity under the influence of radium radiations which acts like a poison in that it produces the apparent abnormalities, and that this substance is intimately connected with the nucleus. But to call this substance a 'contagium vivo' and to endow it with the property of producing the observed results does not aid in the solution of the problem. The solution must be fundamentally chemical in nature, even though the precise reactions involved cannot now be discovered.

The following investigation was made to find how the early development of the egg is affected by radium radiations when (1) the sperm is exposed; (2) when the egg is exposed; and (3) when the egg is exposed immediately after insemination. The problem was suggested by Prof. T. H. Morgan, to whom I take pleasure in expressing my thanks.

#### METHODS

*Nereis limbata* was used in these experiments. In its sexual phase it can be obtained in great numbers during the summer months at Woods Hole, Massachusetts. This material has a great advantage in that practically 100 per cent of the eggs segment and develop normally under appropriate conditions, showing great regularity in the rate of development. The sexually mature individuals swarm in great numbers at the surface of the water on dark evenings, and can be kept, with proper precautions for many hours before they discharge their sexual products. For each experiment the eggs of two females were used. It was found necessary to work with small quantities of eggs since the jelly which the eggs extrude at the time of insemination is very mucilaginous and tends to mat together, in which event the eggs fail to develop normally. To prevent this it is necessary to stir them frequently during the first fifteen minutes, at the end of

when the jelly loses its sticky character. The eggs were killed in Meves' modification of Flemming's fluid. The sections were cut  $5\ \mu$  in thickness and stained with iron hematoxylin.

#### THE NORMAL DEVELOPMENT OF NEREIS

The normal fertilization of *Nereis* has been described by Lilie ('12) so that an extended account here is unnecessary. My observations are in every respect in accord with his. The following is a greatly abridged history of the fertilization phenomena in which only the more important events are mentioned.

As soon as the successful spermatozoön is implanted in the egg membrane it loses its motility and remains exterior to the egg for about fifty minutes. Very shortly after its implantation the cortical zone of colloidal material in the egg begins to be extruded, forming a thick jelly which, as it flows out, pushes away all the spermatozoa except the implanted one. The surface of the egg just beneath the perforatorium of the sperm now rises up in the form of a cone (fig. 1). The fertilization cone thus formed soon sinks, drawing with it the sperm which consequently comes to lie in a depression of the vitelline membrane. The cone, in the meantime has taken a deep stain and is a conspicuous object at the periphery of the egg. About fifty minutes after insemination the cone begins to move inwardly toward the center of the egg pulling with it the sperm head, which, as a consequence of being drawn through the membrane, assumes a band-like appearance. (fig. 2). The middle piece and the tail are left behind. The cone, followed by the sperm head, now moves deeper into the egg and revolves through  $180^\circ$  so that the sperm head is in front. The cone is left behind at this stage while the sperm nucleus moves forward. At this time a sperm aster develops (fig. 3). Later this structure divides unequally, the two asters thus arising forming the cleavage asters. As the sperm head advances the chromatin becomes vacuolated and later breaks up into the haploid number of karyomeres. These gradually fuse, the chromatin spinning out into a delicate spireme. At this time the sperm nucleus is ready to fuse with the egg nucleus (fig. 4).

In the meantime, the polar bodies have been extruded and the egg nucleus, at first very small after the running together of the vesicular chromosomes remaining after the second maturation division, now enlarges. The chromatin forms at first into karyomeres, just as in the case of the sperm nucleus, and then spins out into a delicate spireme. The two germ nuclei now fuse. The spiremes thicken and break up into chromosomes, which, after the first cleavage, become vesicular and finally run together forming the typical resting nucleus of the blastomeres.

The early cleavage needs no description, but a few typical stages that have been chosen arbitrarily for purposes of comparison with the abnormal larvae will be mentioned. In ten hours (depending somewhat on the temperature) the embryo acquires the protrochal band of cilia and begins to swim about actively. Green pigment at the posterior end develops in about fifteen to eighteen hours. In thirty five hours reddish brown pigment develops just back of the protrochal ciliary ring. A little later the first setae appear. At sixty hours the setae are in two pairs and soon after become jointed. The third pair also develops at about this time. One hundred hours after fertilization the palps are formed, and the whole embryo is markedly segmented.

#### EXPERIMENTAL

##### *a. The fertilization of normal eggs by radiated spermatozoa*

The spermatozoa of *Nereis* show remarkable vitality under very adverse conditions. If they are taken from a freshly caught, vigorous male and kept without admixture with sea water, they will remain alive for upwards of fifteen hours, and at the end of that time will fertilize fresh eggs in a normal manner, the embryos arising from such a union showing no marked abnormalities. They can live for a short time in practically fresh water, a fact that renders the sterilization of pipettes a troublesome process. If pure sperm be diluted with sea water the spermatozoa die within a few hours.

The fresh spermatozoa, as free from sea water as possible, were exposed by putting a small drop of the fluid in a thin walled glass

vial of such a size that it exactly fitted into the depression in the capsule in which the radium salt was held. The radium in this experiment was equal to 4 mg. of the pure bromide. The drop of spermatozoa was so spread out on the bottom of the vial that it formed a thin layer, all parts of which must have received equal radiation. A control experiment was made by putting another small drop into a similar vial which was placed near the other, but screened from it by two sheets of lead. As a rule, the spermatozoa were radiated for at least twelve hours, for it was found that shorter exposures produced very slight effects. After so long a treatment the motility of the spermatozoa seemed to be unchanged. Indeed they showed no signs whatever by which to distinguish them from the control or from perfectly fresh spermatozoa. These results are in accord with the statements of G. Hertwig ('12) in regard to the spermatozoa of sea urchins.

The phenomena of fertilization that can be observed in the living egg are normal. Great numbers of spermatozoa collect at the periphery of the eggs and can be seen to be pushed away by the gradual outflow of jelly which forms as soon as the successful spermatozoön is implanted in the egg membrane. If india ink is added to the water it becomes evident that no jelly is extruded at the point of attachment, the spermatozoön appearing to lie at the apex of a cone of ink which marks the region where the jelly is lacking. The time elapsing between the addition of the sperm and the outflow of jelly appears to be normal. Soon after the implantation of the spermatozoön the fertilization cone develops, reaching up to the surface of the egg membrane just below the point of attachment. The further progress of the fertilization phenomena can be seen best in sections.

Eggs killed thirty minutes after insemination show the spermatozoön, entirely normal in appearance, still external to the egg, with the perforatorium extending through the egg and into the substance of the fertilization cone. A little later the cone is retracted, pulling with it the sperm head and egg membrane so that the latter forms a small depression in which the former lies. Then the sperm head begins to be drawn out, penetrating further into the cone, and developing about its perforatorium

the usual attachment granules. The cone itself develops normally, and its behavior in drawing in the spermatozoön is normal.

When the sperm head is in the process of being drawn in by the fertilization cone the first abnormality makes its appearance. The chromatin of the head, instead of forming an unbroken band, as in the normal process may now become broken up into irregular masses (fig. 6). Occasionally, in the normal sperm head as it is drawn through the egg membrane, one finds some evidence of segmentation, but in no case is there so marked a breaking up of the chromatin as is found here. About 10 per cent of the eggs show this abnormality.

From this stage the subsequent behavior of the sperm heads may differ. The further entrance and development of the sperm nucleus may be normal, or the sperm may entirely fail to gain further entrance. In the first case, the sperm head, preceded by the fertilization cone, penetrates further into the egg, revolving meanwhile. After complete revolution the sperm aster develops. The division of the sperm centrosome was found in only a few instances, but was normal when found. The chromatin becomes vacuolated and later forms into the haploid number of karyomeres. At this stage it may normally fuse with the egg nucleus, or else undergo a curious development which results in its failure to fuse with the latter. When fusion occurs the cleavage nucleus divides normally with twenty-eight chromosomes present. There is, then, either a complete fusion with subsequent normal division, or else no fusion at all, and no division of either germ nucleus.

In the latter instances the germ nuclei remain in the karyomere stage. The sperm asters which at first are present now gradually dwindle and finally disappear altogether. Figure 7 illustrates this point. The two asters are hardly as large as the smaller of the original sperm asters. At this time the egg aster has normally disappeared. This case and others to be described are not due to polyspermy, for such eggs are readily recognizable. The number of karyomeres present also shows that only one sperm has entered. Figure 8 shows an egg in which no trace of astral radiations can be found. There are many such eggs in the preparations. The action of the radiation on the sperm, then, has been to prevent

not only its own development, but that of the egg nucleus also. The number of vesicles and karyomeres differ considerably. Normally there should be at most four or five vesicles with twenty-eight karyomeres. Frequently the number of vesicles may be as high as twenty. The appearance of such eggs recalls the condition described by Lillie ('02) in the fertilized egg of *Chaetopterus* which had been treated with KCl solutions of varying concentrations. He found that the nucleus broke up into many fragments which were distributed irregularly through the protoplasm, and were never gathered up into a single nucleus again. Each particle of chromatin was surrounded by a vacuole of liquified protoplasm. The appearance is similar in many respects to that found in *Nereis*, although the mode of formation of the vacuoles is entirely different. Whether both of the germ nuclei are concerned in this process or only one, cannot be definitely settled. The presence of no extra asters shows that if the sperm nucleus has divided the chromatin only has been concerned. In view of G. Hertwig's experiments on the sea urchin, in which the egg nucleus divided without fusing with the sperm nucleus, it seems reasonable to suppose that in *Nereis* also the egg nucleus divides by itself. But here the division is very abnormal, being due to something evidently brought in by the sperm.

The second class of abnormalities is caused by the failure of the sperm nucleus to enter the egg. There is always a fairly large number of abnormalities of this type in material treated in the way indicated, and each egg presents practically the same appearance. It should be mentioned at the outset that this condition is found, though rarely, in the controls.

Although the spermatozoa that have been radiated are as active as those in the controls yet some of them fail to effect a complete entrance into the egg. The actual attachment of the spermatozoön is undoubtedly due to its own activity, but its subsequent entrance into the protoplasm of the egg is due to the activity of the egg itself. It may be inferred, therefore, that the sperm does not, in these cases, call out the proper stimulus for the complete reaction. The egg is able to draw the sperm head in for a short distance only. The details of the entrance of such a sperm are

as follows: The moment the perforatorium becomes implanted in the egg membrane there occurs the usual outflow of jelly. Beneath the perforatorium there forms the usual darkly staining area of protoplasm. The fertilization cone rises to meet the point of attachment, then sinks, pulling the sperm head with it. But as the chromatin is pulled out it is seen to be broken up into irregular masses, a condition not seen in the controls. In some instances the band appears to be continuous, but arranged in a bead like fashion. Thus far, then, the effect of the spermatozoön on the egg has been merely to fail to elicit the full response by which it should be engulfed in the egg.

During this period the egg gives off the two polar bodies in a normal manner. But when the egg nucleus begins its reconstruction, it fails to develop in the usual way. The chromosomes increase somewhat in size, and each becomes surrounded by a deeply staining, granular matrix. The chromatin appears normal. The matrix stains about as deeply as the fertilization cone. Each chromosome, surrounded by the matrix, lies in a clear, non-staining vacuole. Occasionally they can be seen to be segmented, a condition not seen in the control. In such eggs there is a total absence of astral radiations. The egg aster disappears very early, and the sperm aster never develops because the sperm is still entirely exterior to the egg.

The development of the egg nucleus in such a manner is not due to any poison injected into the egg by the radiated spermatozoön, but rather to the failure of the sperm head to enter. That this is true may be inferred from the experiments on partial fertilization made by Lillie ('12). These consisted of centrifuging the fertilized egg at such times that the attached spermatozoön, still external to the egg membrane, was removed wholly or in part. In the former case, when no chromatin was introduced, the eggs extruded the jelly and extruded both polar bodies. But the egg nucleus, instead of forming a typical vesicle with karyomeres, failed entirely to develop, the chromosomes lying free in the protoplasm. Around each chromosome was a darkly staining matrix, evidently corresponding to the chromosomal vesicle, through no vesicular wall was formed. This condition is an



exact picture of that found in eggs in which the radiated spermatozoön has failed to enter.

It is evident, then, that the radiation of the spermatozoa is not responsible for the peculiar development of the egg nucleus. Nor is the failure of the sperm to set up an appropriate reaction in the egg which should ensure its entrance an effect of the radiations per se. For the same condition is occasionally found in the controls. Probably any treatment which changes the spermatozoa would bring about the same result.

To sum up the results of this experiment, it may be said that the abnormal spermatozoa can be divided into two classes, according to the reactions they initiate in the egg. Those of the first class are able to activate the egg normally and are consequently drawn into the egg protoplasm; those of the second class are able to produce this result only in part. The spermatozoa of the first class induce the normal extrusion of the jelly, and the formation of the fertilization cone, but they fail to develop the normal cleavage asters and fail to fuse with the egg nucleus. The second class of spermatozoa bring about the normal extrusion of the jelly, and the formation of the fertilization cone, but cannot stimulate the egg sufficiently to cause it to draw them in. This defect results in the abnormal development of the egg nucleus. It cannot be said that these spermatozoa are injured in the chromatin alone unless it is held that any process that weakens their vitality acts specifically on the chromatin. It is apparent, however, that the chromatin is actually injured. This experiment shows that when failure of the nuclei to fuse occurs, the egg does not develop at all, thus differing radically from the sea urchin egg under similar conditions.

*b. The subsequent development of eggs fertilized by radiated sperm*

Cell division in the majority of eggs is normal, although usually somewhat delayed. The chromosomes split in the normal manner and form vesicles at the telophase. Later the vesicles fuse to form the resting nucleus. No irregular mitoses were

found. Those eggs in which the germ nuclei fail to fuse do not divide.

The first striking abnormality in the growing embryos appears when they are about twenty hours old. At this time the pro-trochal band of cilia and the green pigment at the posterior end are wholly lacking. Such embryos are of normal shape, but remain motionless, while the others in the dish are swimming actively about. In twenty-eight hours some are still lacking in ciliation, while others have developed the ciliary ring, which shows characteristic abnormalities in the distribution of the cilia. Instead of being in a continuous band, they occur in patches scattered irregularly. Such embryos swim slowly, and with a curious uneven motion, pursuing a devious course. In forty-eight hours, when the control embryos are well provided with green and red pigment and swimming vigorously at the surface, the radium embryos are still unpigmented, or else have the pigment broken up into small patches. The ciliation is still abnormal. The motion is consequently abnormal, the embryos tumbling over and over, or taking a spiral course, turning on their long axis. In seventy-eight hours a few of the embryos develop setae and pigment, but both are abnormal. The setae rarely grow out to full length, and are irregularly distributed. The majority of the embryos that have survived as long as this remain in about the same condition that they showed at thirty hours.

This mode of development occurs occasionally in the controls, especially if the conditions of growth are unfavorable, but never in as large proportions as among the radium embryos. Unfavorable conditions obtain if many eggs are allowed to develop in a small amount of water, or if they become matted together after fertilization. If eggs are compressed under a coverglass during early cleavage they develop precisely the same type of abnormalities.

In these embryos the effect of the injured spermatozoön remains latent for many hours, and is displayed for the first time when the embryo begins to differentiate the pigment and the cilia, and begins to grow notably in size.

*c. The development of eggs radiated before fertilization with fresh sperm*

In the second series of experiments the unfertilized eggs were placed in a few drops of water in a watch glass. The glass tube containing the radium was about 1 cm. in diameter and 1.5 cm. in length. When this tube was set down into the water, the surface tension of the water was sufficient to draw the eggs up along the sides of the tube, thus distributing them fairly evenly, and ensuring an equal amount of radiation. Control eggs were treated in the same way except that the glass capsule, similar to the radium capsule was empty. In all of these experiments small quantities of eggs were used.

The unfertilized eggs, after two hours exposure to the radium rays show no change in appearance. The germinal vesicle and the alveolar layer are intact. High magnification shows no alteration in the protoplasm, or in the oil and yolk spheres. When such eggs are inseminated with fresh sperm many immediately throw off the jelly in a normal fashion. As a consequence the alveolar layer quickly disappears. The jelly is apparently normal in consistency, and loses its mucilaginous character in about fifteen minutes, just as with normal eggs.

A considerable number of eggs, however, fail to extrude the jelly at once and consequently cannot push away the great quantity of spermatozoa that surround them, as can the normal eggs. Polyspermy results in such cases, many eggs being penetrated by a dozen sperm heads. Sections of such eggs show the alveolar layer still intact (fig. 9). This layer presents a fairly normal appearance, with alveoli closely crowded together, but not always radially arranged. Typically it extends entirely around the egg, and is about six or seven  $\mu$  in diameter. But in these eggs it is very unevenly distributed, being sometimes absent altogether from one side, and massed in a thick layer on the other. There is no definite place at which the layer collects in these cases.

The eggs showing this peculiarity are uniformly larger than normal, the increased diameter being due to the greater width of the alveolar layer which varies from eight to fifteen  $\mu$  in thickness.

When massed at one point it exceeds this limit. The explanation for this phenomenon is not known. According to Lillie, the extrusion of the jelly takes place when the spermatozoön becomes implanted. As the jelly diffuses outward sea water passes in to take its place, so that the original size of the egg is maintained. If this is true, it may be that the sea water has entered faster than the jelly has been extruded, and consequently, the whole layer increases in width. However, this does not explain the fact that the alveolar layer is unevenly distributed.

The increased size of the cortical layer does not persist as measurements show. Within seventy minutes after fertilization it has decreased noticeably, and after eighty minutes has disappeared altogether. The following figures are average measurements of a large number of eggs.

	<i>Normal</i>	<i>Radiated</i>
45 minutes after insemination.....	106 × 92 $\mu$	115 × 105 $\mu$
60 minutes after insemination.....	105 × 87 $\mu$	111 × 95 $\mu$
70 minutes after insemination.....	105 × 90 $\mu$	107 × 95 $\mu$
80 minutes after insemination.....	104 × 91 $\mu$	105 × 94 $\mu$

It is thus seen that before the first division, which occurs in about ninety minutes, the eggs are of normal size. Sections show that the alveolar layer by that time has entirely disappeared, except in the few cases in which fertilization did not occur.

In the control series the alveolar layer is extruded at once and nothing is seen of it inside the egg forty-five minutes after insemination. The germinal vesicle also breaks down soon after the extrusion of the jelly. I found no exception to this. But in the radiated eggs the vesicle shows some curious modifications in behavior. It may break down at once even though the alveolar layer is still present, or it may remain intact for a considerable period. In the former case, the chromatin, which, during the process of dissolution of the nuclear membrane has collected in the form of chromosomes, is left free in the protoplasm. If the vesicle remains entire, the chromatin, which is now in granules, collects along the periphery, just beneath the membrane. This is the normal condition for it at this period. Occasionally the granules collect in the form of chromosomes which are scattered!

through the nucleus suspended in a delicate non-staining network. After a time (varying in different eggs from forty to eighty minutes) the vesicle breaks down, and the subsequent history of the chromatin is the same as in the case of eggs in which it disappeared at once. Its dissolution is apparently independent of the presence of the alveolar layer. Here, then, is a case in which the chromatin is not visibly affected by the radium radiations but the protoplasmic activities are much modified, although the structure of the protoplasm itself remains unchanged in appearance. This fact is in opposition to Hertwig's view that only the chromatin elements are affected by the rays.

The mode of entrance of the spermatozoön into eggs which retain their alveolar layer is considerably modified from the normal. The fertilization cone rises up under the point of attachment of the perforatorium as usual, and later sinks into the egg, pulling the sperm head with it. But it takes no stain at all. Figure 12 shows an egg killed forty-five minutes after fertilization. The spermatozoön has become implanted in the egg membrane, and has begun to penetrate a short distance into the alveolar layer. No cone is seen. A later stage is shown in figure 10 in which the alveolar layer is still intact and is wider than normal. The sperm heads have penetrated nearly their entire length. They never completely enter the protoplasmic area of the egg, but remain in the alveolar layer until it is extruded, at which time some of them continue their course inward. Intermediate stages in this process have not been found. It may be inferred that the sperms enter entirely since at the time that the cortical layer is extruded (in about eighty minutes) none can be found exterior to the egg, while within many may be found in various stages of development.

The process of maturation in those eggs which fail to extrude the alveolar layer at once is much modified. The germinal vesicle disappears normally in the great majority of cases. The chromatin, now free in the protoplasm, may move toward the animal pole where the spindle normally should form, or it may remain in the interior of the egg. Figure 11 shows a case in point. The chromosomes are ill-formed, show no particular arrangement

and are deep in the egg. Careful search has not revealed any asters or spindle fibers. In figure 12 the spindles have formed, as well as the chromosomes, but the asters are lacking. Here, too, the spindle forms in the center of the egg. There are other cases in which very small asters are present, with fairly normal chromosomes. Occasionally figures like figure 13 occur, in which the achromatic portion of the figure is normal, but the chromosomes are segmented. A more normal type is seen in figure 14. The normal condition seems to be due to the fact that the alveolar layer has been extruded at the periphery nearest to the spindle, allowing the usual relation to obtain between the protoplasm and the sea water in the egg protoplasm.

In a majority of cases, however, the alveolar layer is extruded normally. In all instances the spermatozoa may enter normally or may show a characteristic abnormality, which, curiously enough, is similar to a condition found in normal eggs fertilized by radiated sperm. It consists in the failure of the fertilization cone to stain the usual dark color. Figure 15 shows such a condition. It will be noticed that the sperm is normally implanted and has already become anchored in the substance of the cone by minute attachment granules. The cone itself is transparent, and traversed by a delicate achromatic network. Immediately around it the protoplasm is of normal appearance. Later the cone sinks into the protoplasm in the normal manner, pulling with it the sperm head. The non-staining quality apparently has no effect on its function.

Lillie suggests that the normal deep stain of the cone is due to a coagulating fluid injected by the sperm into the egg through the perforatorium. If this is true we have another evidence that the chemical reactions of the egg protoplasm, have been changed by the radiation.

The formation of the first polar body may be very abnormal, but the abnormalities concern chiefly the achromatic portions of the spindle. As a rule the chromatin is but slightly affected. Figure 16 shows a well marked tri-aster with large centrosomes which are abnormal in size. It will be noticed that the chromosomes, even in the minutest details of splitting are perfectly nor-

mal. This condition can hardly be interpreted as a first and second polar division combined, since it occurs much earlier than the normal time for the latter. The distribution of the chromosomes shows also that the two lower asters do not represent the second spindle. The sperm is still exterior. Figure 17 is more normal, having but two asters. The spindle has not revolved, as it should have done at this time. The normal figures are at the telophase of the first polar spindle. The asters are of normal size and appearance, and the chromosomes show no abnormality of any sort. It is curious that they have taken up a position outside of the spindle, more or less in the place which they should occupy if the spindle had revolved.

There are many other abnormalities in the formation of the spindle, but the examples given are typical. In practically no case in which the spindle forms do the chromosomes show any abnormality.

The second polar spindle may be entirely suppressed or may develop abnormally in several respects. When the first polar body is not extruded, the second also may fail to be given off. Figure 18 shows what is evidently a second maturation. The egg was killed eighty minutes after insemination, at a time when the control eggs had already extruded the second polar body, and possessed a well developed egg nucleus. The spindles are normal in general appearance, but abnormally small, and placed in the center of the egg. It is difficult to say what would be the future history of such an egg. Evidently the polar bodies will not be extruded. Figure 19 shows a similar case except that the spindles are larger. It will be noticed that one of the sperm asters has formed before the revolution of the sperm head and cone, an unusual occurrence.

It is evident, then, that the injury done by the radium has affected both the chromatin and the protoplasm, particularly the latter. When the alveolar layer is not extruded the astral systems are abnormal and consequently the chromosomes. In such cases it is difficult to say how far the chromatin has been injured.

After the maturation period the remaining chromatin collects in one or more large vesicles of normal appearance. Occasionally the number of such vesicles may be very large and the karyomeres very numerous. The extra number may be due to the fact that the polar bodies have not been extruded, so that there is much extra chromatin. The subsequent behavior of these vesicles differs accordingly to whether they fuse with the sperm nucleus or not. In the former case the fusion may be entirely normal, and the subsequent division perfectly regular. Or the cleavage nucleus may never divide, but, on the contrary, increase considerably in size until it is nearly half as large as the germinal vesicle. The chromatin, in the meantime, breaks up into minute granules and passes out from the nucleus into the surrounding protoplasm. Figure 20 shows such an instance. The nuclear wall displays no breaks through which the granules might have passed out bodily.

Such a condition might be explained on the basis that the egg chromatin had been injured by the radiations and had formed, as a result, a poison, which affects the sperm chromatin. The general effect of the radiation is to cause the chromatin to break up into granules. This is the explanation suggested by G. Hertwig.

If the sperm nucleus does not fuse with the egg nucleus it fails to develop past the spireme stage, and in many cases does not develop even as far as that. Up to this time it has behaved normally. Even when many sperms enter (fig. 19) each one pursues the normal course in the egg. In figure 21 are shown two sperm nuclei in several separate vesicles, in which the karyomeres have fused and commenced to draw out into spiremes. Which vesicles belong to the egg and which to the sperm nuclei is difficult to determine since they have become exactly the same in appearance. In such a case, if a poison has been generated, it has failed to produce any marked effect on the egg chromatin itself. The only apparent abnormality is the failure of the nuclei to fuse.

Division of the egg may occur without the appearance of asters and without division of the chromatin. Figure 22 shows an egg in which all the chromatin is in one blastomere. Figure 23 shows the protoplasm dividing without any division of the chrom-



atin. The vesicles, which have not developed into the spireme stage, are scattered irregularly about in the protoplasm. The large number of vesicles, with forty-two karyomeres, and two asters, indicates that two sperm nuclei are present. But they have ceased to develop and their astral systems have failed to develop normally. This failure is evidently not due to polyspermy for polyspermic eggs develop in a different and characteristic manner.

To sum up: The effect of radium on the unfertilized eggs is seen after fertilization with normal sperm in both the protoplasm and the chromatin. The failure of the surface layer to be given off is due to a change in its constituents under the influence of radium. This failure leads to abnormalities in the formation of the polar bodies. The chromatin at first displays very little evidence of injury. Later, however, the injury shows in the failure of the egg nucleus to develop properly. The sperm nucleus also fails to develop in these cases.

*d. The development of embryos from radiated eggs fertilized  
by normal sperm*

The later development of these eggs is not different from that of the normal eggs fertilized by radiated sperm, but the proportion of unfertilized eggs is greater, and the rate of development is slower. This may be inferred from the fact that the sperm, in many cases, cannot enter the egg for more than an hour after insemination because the cortical layer is still present. Occasionally some eggs divide into three or four parts at once, or, if into two blastomeres, the relative sizes of the two are abnormal. The subsequent development is also greatly retarded. When the control embryos are already in the early trochophore stage, these have not yet acquired the protrochal ring of cilia. A large proportion die before they reach the trochophore stage. Whether the proportion of abnormalities is greater in this series than in the first is hard to say, since always in the controls, a few embryos are abnormal. The type of abnormality that develops is precisely the same as that described in the preceding section.

*\*c. The development of eggs normally fertilized, and exposed to radium*

In this series of experiments, the eggs were inseminated and stirred frequently for five minutes, then put over the radium capsule, which had an activity equal to 4 mg. of the pure bromide. At intervals a small quantity was taken out, half of the eggs being killed, and half put into sea water and allowed to develop.

The early stages of fertilization are normal in all respects. The jelly is extruded at once (this began before the eggs were exposed to the radium) and consequently only one sperm enters. The external phenomena of implantation of the sperm and the rising up of the entrance cone are normal. A study of the sections shows that in many cases the fertilization cone does not take the usual dark stain, but remains as a very light, nearly transparent vesicle, similar to that described in the second series of experiments. (fig. 15). The sperm enters normally, however, as in the former case. In the meantime the polar bodies are normally extruded.

The first indication of abnormal development is seen in the behavior of the germ nuclei. In many instances these do not fuse, but remain separated, at some distance from each other and independently develop chromosomes and normal spindles. Figure 26 shows an early stage of the process. The nuclear walls are still intact. The sperm nucleus has not developed as far as the egg nucleus, being still in the closely wound spireme stage. The egg nucleus with its aster has moved from its position under the polar bodies, away from the approaching sperm nucleus. Figure 24 is a later development. Probably the structure nearer the polar body is the sperm nucleus which has failed to develop much further than the stage shown in the preceding figure. The egg nucleus, however, has lost its nuclear membrane and has formed distinct chromosomes which are not as clear cut as normal, but show some evidence of becoming broken up into granules. Figure 25 shows a still later development. The nuclei have migrated as far from each other as appears possible and lie between complete and normal asters. In each nucleus the haploid number of chromosomes can be determined. In figure 27 the division has

gone still further. Judging from the size of the chromosomes, it seems probable that they have split, fourteen daughter chromosomes going to each pole; but the number could not be exactly determined. The chromatin in this case is perfectly normal. In the instances mentioned, and in many others that have been observed, it is apparent that the plane of one spindle does not coincide with that of the other or with the first normal cleavage plane.

Occasionally, when the nuclei fail to fuse, the astral systems do not develop, or, if they do, remain only a short time. The chromatin in such cases degenerates, forming irregular, granular masses which lie free in the protoplasm (fig. 28). One germ nucleus has evidently degenerated faster than the other and its chromatin has become much scattered and takes the hematoxylin stain very lightly.

When the fusion of the nuclei occurs the process appears to be normal. Abnormalities appear when division begins. As a rule the chromatin shows few signs of injury, but the astral systems are abnormal. Very commonly a tri-polar figure arises (fig. 29). The asters are normal in appearance and the centrosomes are normal. The chromosomes are very irregularly arranged, as would be expected. But each one is well formed, showing no evidence of degeneration.

Frequently, however, the chromatin displays undoubted signs of injury, as shown in figure 30. Not only are the chromosomes indistinguishable but some of the strands are now broken up into minute granules. There are all gradations between such a condition and a normal figure of which there are very few in the material.

The effect, then, of the radium on fertilized eggs is much more marked than that on the unfertilized eggs. This accords with the results of O. and P. Hertwig, who found that the embryo frogs arising from radiated fertilized eggs showed more marked abnormalities than those in the other series. The effect on the chromatin are similar in some respects to those described by P. Hertwig in the egg of *Ascaris*. It is evident, however, that the protoplasmic activities of the eggs of *Nereis* are almost equally affected

with the chromatin, and that the fertilized eggs in the two cases respond in very different ways.

*f. Subsequent development of the embryo*

The great majority of the eggs do not divide. In some cases they may divide into three or four blastomeres at once, as would be expected from the division figures already described. Very few of the eggs continued to divide after ten hours of development. A small number of early trochophores developed but no pigment formed in them, and the cilia were abnormal. No embryos survived after twenty-four hours.

*g. Development of eggs radiated before and after fertilization with normal sperm*

The early phenomena of fertilization in eggs radiated for two hours before fertilization and for varying periods *after fertilization*, do not differ to any extent from those described in the second series. In some lots of eggs the alveolar layer is extruded normally in almost every instance, while in other lots it is retained for some time after the implantation of the sperm. In the latter case polyspermy invariably occurs, as many as ten sperms entering at practically the same time and developing at the same rate.

The mode of entrance of the sperm in either case is the same as that described in the second series. In many instances the fertilization cone is an almost transparent vesicle. But, as in the previous cases, this change in the staining reaction does not indicate any change in function, for the spermatozoön is drawn in normally, and the whole process is without any other indication of abnormality. The further development of the sperm head is normal. The head and cone revolve, and an aster forms at the base of the head. The chromatin, as the head enlarges, collects into a few karyomeres. But the number of vesicles which contain these structures may be very large. In some eggs there may be as many as forty, indicating that each sperm has broken up into at least four. Their subsequent development will be described after the behavior of the egg nucleus has been traced.

The germinal vesicle breaks down as a rule, within a few minutes after insemination, even though the alveolar layer is still present. The egg centrosome divides normally, the daughter centrosomes migrating to the opposite sides of the group of chromosomes which lie free in the protoplasm without any definite arrangement. If the alveolar layer has been extruded the spindle usually moves to the surface where it develops normally in many cases. In such instances the chromosomes are not at all affected, but split into the normal crosses and rings with great precision. The achromatic figure also appears to be normal. When the alveolar layer is not extruded at once, and, in some cases, even when it is, the whole first polar figure remains near the center of the egg. Judging from the scarcity of extruded polar bodies, it is probable that the polar body is not often extruded.

There are, however, several marked departures from the course outlined above. Figure 31 shows a condition found in several eggs. There is but one centrosome and no real aster. The spindle fibers are normal and attached to the chromatin masses. No normal chromosomes are present, the chromatin remaining either as a long narrow thread, or as irregular, homogeneous masses. In each instance it is in two distinct groups separated by a varying distance. The size of the chromatin thread indicates that splitting has not occurred in the right hand group. In the other group the chromatin is in very irregular masses of unequal size. Figure 32 is a later stage of the same condition. In this egg there is a normal sperm head and cone already revolved, but the sperm aster is not apparent. Some of the chromosomes are fairly normal, while others are mere masses of chromatin. The separation of the groups and of the spindle fibers attached to them is a curious phenomenon. In some instances both polar bodies are given off, but in many cases none at all are extruded.

During this time the sperm heads have developed up to the vesicular stage, as has been mentioned above. Their further development may proceed along different paths. In some instances the karyomeres apparently break down and fuse together, taking the form of fine spireme, or even breaking up into chromosomes. If several sperm heads enter there are at least as many

asters, and in many cases, a much greater number. But such asters are not connected with each other by spindle fibers nor are they centers for chromatin masses. In some instances a great number of asters is present but their origin is uncertain. If we assume that each original sperm aster has divided normally, it would be necessary that fifteen sperms entered the egg about at the same time. This number is greatly in excess of any observed case. The extra asters may have arisen *de novo*.

Perhaps the most common abnormality is the presence of a great number of vesicles without any aster. In view of the facts just presented, namely, that sperm asters develop when polyspermy occurs, it is evident that the extra vesicles represent a single sperm nucleus that has divided, or else the egg nucleus, or finally, sperm nuclei that have lost their asters. Figure 33 is a case in point. The only aster present is extremely small. The vesicles, of which there are a great number in the egg, are still intact although the karyomeres have about disappeared, having gone into the spireme form. At this stage we should expect the germ nuclei to fuse but in this case, one hundred minutes after insemination, when the control eggs have already divided, we find no evidence of fusion. Such eggs do not divide but show the very common phenomenon of budding.

By many observers this phenomenon has been described in eggs that are rapidly degenerating. In the eggs of *Nereis*, at about the time when cleavage should take place, the protoplasm at the periphery separates off into buds of irregular size and shape. They contain no chromatin unless by chance some of the vesicles are in the region where the budding occurs. The protoplasm of the buds and the region directly contiguous shows marked signs of degeneration, appearing to be more fluid and possessing only a few protoplasmic granules, instead of the closely crowded quantities present in the more normal regions.

The general effect of the radium radiations on the eggs treated in this manner seems to be more on the protoplasm than on the nucleus. The surface layer is greatly affected and as a consequence may fail to extrude the alveolar layer. When division would normally occur, only a budding takes place. If the sperm

asters are developed from the egg protoplasm, it is evident from their peculiar behavior that the latter substance has been injured. The chromatin also is affected, as is shown in the cases of irregular formation of the maturation chromosomes, and in its behavior in the nuclear vesicles. But the many cases of perfect formation of the chromosomes and their normal splitting, even in the minutest details, indicate that the effect of the radiations on the chromatin has not been profound.

#### DISCUSSION

The work of G. Hertwig and others points to the conclusion that the chromatic elements of the nucleus are the only parts of the cell which are disturbed by the radium radiations. The conclusion that no other constituents are affected has been drawn from the fact that the usual methods of technique do not reveal any chemical changes in the protoplasm. Such a criterion is, in many cases the best we have, but it is evident that staining will not reveal all of the cell constituents which are known to be present, which, like enzymes, operate on the protoplasm of the cell, changing its chemical constitution; nor will it give evidence of the nature of such changes.

There are no visible changes in the radiated sperm—a fact that led Hertwig to believe that the protoplasm was uninjured. But I believe that in the sperm of *Nereis* there is evidence that a protoplasmic change has taken place. This change becomes apparent through the activities of the egg in the early stages of fertilization—activities which are incited by the stimulus of the sperm itself. In the first place, when the sperm becomes implanted in the egg membrane it injects into the egg a coagulating substance which, according to Lillie, changes the protoplasm so that it takes a fairly deep stain with hematoxylin. This staining reaction is frequently lacking. It cannot be said that the egg protoplasm has been changed since all of the eggs used in the experiment were normal. Therefore it may be inferred that the protoplasmic elements in the sperm have been modified.

Secondly, the sperm in many instances fails to gain entrance into the egg. The engulfing of the sperm is undoubtedly due to the activity of the egg, since the sperm is motionless from the moment it becomes implanted in the egg membrane. Such activities are incited by some substance in the sperm itself, and are not due merely to the pricking of the membrane by the perforatorium, for in many instances the implanted sperm does not enter. The seat of such a stimulating substance is not certainly known. Probably it does not reside in the nucleus, since all of activities of the egg, such as the throwing off of the cortical layer, the formation of the fertilization cone, and its subsequent movements, occur while the nucleus is still intact and entirely outside of the egg. It is very likely that the stimulus resides in the head cap of the spermatozoön, and is injected into the egg with the coagulating fluid. The failure of the sperm to enter is due to the injury to this substance and a consequent failure properly to activate the egg. Such an injury is not due specifically to the radium treatment since old spermatozoa, kept fourteen hours in a vial will produce the same results. Probably any treatment which weakens them in any way has this effect. These two instances, I believe, indicate that the sperm protoplasm has been injured by the radium radiations.

That the chromatin of the sperm has been injured has already been pointed out. The difference in behavior between the Nereis sperm and that of the sea urchin, as described by Hertwig, should be mentioned. In the former case, the sperm nucleus fails to develop past a certain point, and fails to fuse with the egg nucleus. The latter structure also fails to develop due apparently to the failure of the sperm centrosome. In the sea urchin, on the other hand, the sperm centrosome develops perfectly, becoming the dynamic center of division for the egg nucleus, which divides by itself as a rule in a normal manner. If the sperm nucleus has migrated into a position close to the spindle it acts as a foreign body in disturbing the normal behavior of the chromosomes. In some cases the two nuclei fuse, but the sperm nucleus is soon eliminated in subsequent divi-



sions. Development is consequently parthenogenetic, a condition which does not obtain in the Nereis egg. It should be noted that the sea urchin egg is an especially favorable subject for parthenogenetic development, while Nereis is not. Consequently the two cases are not exactly parallel and the behavior of the one should hardly be expected to be repeated in the other.

The generally accepted theory that the cleavage centrosomes are derived from the divided centrosomes brought in with the sperm has recently been questioned by Lillie who believes that in Nereis the centrosome appearing at the base of the sperm head is derived through the interaction of the sperm chromatin and the egg protoplasm. The fact that the middle piece is always left behind on the outside of the egg membrane seems to support this view. A discussion of the theory is not in place here, but it may be pointed out in passing that this fact alone is not conclusive evidence that no formed centrosome has been introduced by the sperm, since in many insect spermatozoa the centrosome is contained in the sperm head. If, however, in Nereis, the centrosome is a product of the sperm chromatin and the egg protoplasm we have evidence that the sperm nucleus has been injured. If, on the other hand, the older view as to its origin is true we have evidence that the radium radiations have affected a protoplasmic structure.

A discussion of the effect of radiations on the unfertilized egg raises some interesting questions concerning certain protoplasmic activities of the egg. It has been shown that in many cases the cortical zone, composed largely of colloidal material is not extruded at the time of implantation of the sperm, but remains for a considerable period within the egg, and is given off slowly during subsequent development before cleavage. As a result polyspermy occurs. The extrusion of the surface layer is due, according to R. S. Lillie, to the sudden increase of permeability of the plasma membrane, resulting in an outflow of substances previously impermeable to it. That the membrane is rendered somewhat permeable at the time of implantation of the sperm is evident from the fact that water is taken up, resulting in a swelling of the alveoli. But the normal permeability is

not fully attained. The failure of the cortical layer to be given off may be due to a change in the plasma membrane, or to a change in the colloids of that layer. The possibility of such a change will be discussed later.

The formation of the polar bodies has been shown to be abnormal both in respect to the chromatic and achromatic elements of the spindle. It might be concluded from this that both parts were equally affected by the radium radiations but this is not necessarily true, for it has been shown that the presence of the cortical layer has an inhibiting effect on mitotic phenomena. This suggestion has been made by Bataillon who holds that in the cortical layer are certain katabolic products of metabolism which hold the egg in check until they are given off. The increased permeability at the time of fertilization allows them to be extruded. The resulting egg membrane is therefore a result of such an action and is incidental, indicating merely that certain more fundamental phenomena have been taking place. In *Nereis*, when the cortical layer persists, the maturation divisions are always abnormal, remaining permanently in the interior of the egg or else showing other defects which have been described above. If, however, the cortical layer is extruded in part, the spindle migrates to that region and the figure is normal. It should be mentioned, however, that occasionally in eggs from which the cortical layer has been extruded, the same type of abnormalities may be found. The radium radiations therefore, have affected not only the surface layer, but also the deeper lying protoplasm.

That the abnormalities in the chromatin appear during the formation of the chromosomes, when they are being built up from the protoplasmic constituents indicates that the latter themselves may have been modified, or that the agents through which they are so built are affected, as well as the chromatin itself. These abnormalities are even more strikingly manifested in the division of the fertilized egg, as described above, and also by P. Hertwig.

If we attempt to explain the phenomena already described by the hypotheses of Schwarz or of Hertwig we are at once involved

in difficulties. The latter involves a number of difficult assumptions. First, when the sperm is radiated, the poison generated in the nucleus must remain altogether in that structure, since the protoplasmic activity concerned in movement is not affected, or else it must be assumed that the poison is specific for the chromatin itself, not affecting the protoplasm. And secondly, it must exert no influence on the egg protoplasm during division, since a normal haploid division can occur provided the sperm head does not mechanically interfere in the spindle. If the sperm head fuses with the nucleus its poison exerts no influence on the egg chromatin, other than to cause it to eliminate the sperm chromatin. But it has been shown that in *Nereis* the protoplasm of the sperm has evidently been changed. Hence it would be necessary to postulate that the poison had extended into the protoplasm and had affected the sperm centrosome—a condition contrary to the first supposition. In the egg it is very evident that the protoplasm has been affected. Hence the hypothesis that poison is generated solely in the nucleus is not tenable. A modification of the poison theory will, I believe, explain more of the facts, and such a modification will be discussed later.

The lecithin hypothesis is hardly as satisfactory as the other. The fact that the sperm nucleus is undoubtedly affected by the radiations makes it evident that they are not specific for the lecithin in the protoplasm. The behavior of the radiated egg is more easily explained for it has been shown that the cortical layer, which contains considerable lecithin is affected. If that substance which is distributed throughout the egg is utilized in the upbuilding of the chromatin, the phenomenon could be explained.

These two hypotheses, therefore, fail at certain points when they are used to explain the facts observed in the fertilization of the egg of *Nereis*. I believe that I have given good evidence that both hypotheses may be true in part, since both protoplasm and chromatin are affected by the radium radiations. The question now arises as to the means by which such changes can be brought about.

The solution of the problem lies, I believe, in the fact that the protoplasmic and nuclear elements are not directly affected by the radiations but only indirectly by means of enzymes which are activated by the treatment. In support of this view may be mentioned a number of observations. The experiments of Schwarz ('03) and of Wohlgemuth ('04) prove that lecithin is readily decomposed by treatment with radium. But the latter observer, who investigated the action of radiations on pure organic substances such as olive oil, asparagin, peptone, and finally lecithin, found entirely negative results. In no case were there any observable changes in the substances. From this he concluded that the breakdown of the cells and tissues which contain these substances was due, not to the direct effect on them of the radiations, but to the increased autolytic activity of enzymes in the cells.

The question whether all cell constituents (nucleo proteids, simple proteids, lipins, carbohydrates, and salts) are affected cannot be determined, but an experiment by Wohlgemuth, the results of which are significant in this connection, throws some light on the subject. To test further his hypotheses that the autolytic enzymes are activated by the radium radiations, he exposed portions of tuberculous lung for varying periods, and found that the total amount of nitrogen given off was at first increased fourfold. In the end, however, the control material showed the same total amount as the other portion. But the experiment indicated that the enzymes which bring about a decomposition of the nitrogenous material in the cell are greatly activated. We should look therefore, to the proteins and to lecithin for evidences of decomposition.

That enzymes are activated by radium radiations has been shown by numerous observers. They exert an accelerating effect on peptic digestion, on the diastatic enzymes of the blood, liver, saliva, and pancreas. "This favorable action is not always observable immediately; very often retardation occurs during the first twenty-four hours, this being gradually neutralized, and then replaced, if the experiment is sufficiently prolonged, by an acceleration" (Euler). The promotion of plant growth is also

attributed by Falta and Schwarz ('11) to enzyme activation. Gager ('08) and Congdon ('12) have also found some evidence of acceleration in growth, if the intensity of the radiation is not too great. When *Drosophila* eggs are placed close to a strong preparation of radium, their development is retarded. From these experiments it may be concluded that many kinds of enzymes may be activated but that the lytic enzymes are more stimulated than those that play a synthesizing rôle.

The decomposition of the nitrogenous compounds of the cell is in part normal and in part abnormal. Lecithin breaks down into cholin and finally into trimethyl amine, a reaction which does not take place in life. But the decomposition of the nucleoproteids occurs constantly under normal conditions. Under the influence of enzymes these substances are oxidized to nucleinic acid and finally into some of the purin bases (guanine, hypoxanthine, etc.). The reverse reaction also occurs, by which the complex nucleoproteid is built up again from the simpler materials. The seat of this process is at the nuclear wall, where the nucleoproteids and the protoplasmic proteins adjoin. If, then, we assume that under the stimulus of the radiation the katabolic changes in the nucleoproteids takes place at a more rapid rate than the synthesizing reactions (an assumption that is warranted on the basis of Wohlgemuth's results), we have an explanation of the behavior of the radiated chromatin in breaking up into granules, and failing to divide normally. The acceleration in growth, found by Gager and Congdon when the material was exposed to slight radiation, may be explained on the ground that under such conditions the synthetic processes are stimulated, while under a more intense radiation the opposite reaction obtains.

The behavior of the radiated cells must, I believe, be interpreted in the light of the increased activity of these autolytic enzymes which act both on the chromatin and on the protoplasm. A radiated spermatozoön is affected chiefly in its nuclear constituents which are broken down into simpler compounds. That these are still acid is indicated by their staining reactions. When such a cell enters the egg, the nucleus is unable to develop normally, and to divide with the egg nucleus, because it cannot build up

nucleo-proteids at a normal rate. Hence it is either eliminated, as Hertwig has shown, or remains stationary, after developing up to a certain point. In like manner, the egg nucleus, after the egg has been radiated, fails to develop. That the normal sperm which fertilizes such an egg may develop, as suggested by Hertwig, indicates that the egg protoplasm, made up of "simple" proteins, has not been greatly changed. In *Nereis* evidently such proteins have been affected, as shown by the abnormal behavior of the sperm nucleus. When the fertilized eggs are radiated, the radiation is applied at a period when the synthetic process in the formation of the chromosomes is most active. Hence, any acceleration of the autolytic enzymes results in a speedy deterioration of the chromatin. The latent period which is found in many instances when the radiation is neither prolonged or intense may be explained by this hypothesis. Only a slight acceleration is brought about by the treatment, but the effect of autolysis is cumulative, and becomes manifest after a period, depending on the rate of acceleration. The nuclear material has been broken down to such an extent that, after division, the chromosomes cannot be built up again. In this connection it might be suggested that the normal nuclear division takes place when the ratio between the amount of nucleoprotein and simple proteins of the protoplasm has reached a certain limit. After the division the ratio is changed and constructive action again takes place.

#### SUMMARY AND CONCLUSION

1. Radiated spermatozoa of *Nereis* react in two ways to the normal egg. They may normally stimulate it, and be drawn in, but subsequently fail to develop, or they may fail to stimulate the egg sufficiently and so remain external. In the first case, the sperm nucleus and aster may fail to develop and to fuse with the egg nucleus. In the second case, the egg nucleus develops without an aster.

2. The radiated egg at the time of fertilization may or may not extrude the cortical layer. In either case, the maturation phenomena are more or less abnormal. The germ nuclei develop

abnormally and mitosis does not occur, although the protoplasm may divide.

3. Radiation of the fertilized egg results either in a failure of the fully developed germ nuclei to fuse, or in abnormal division of the cleavage nucleus.

4. Eggs radiated before and after fertilization show very marked evidences of protoplasmic degeneration.

5. In general, both protoplasm and chromatin are affected.

6. The previous hypotheses do not satisfactorily explain these facts.

7. It is suggested that the radium radiations act indirectly on the chromatin and protoplasm by activating autolytic enzymes which bring about a degeneration of the complex proteids, and probably by affecting other protoplasmic substances in the same manner.

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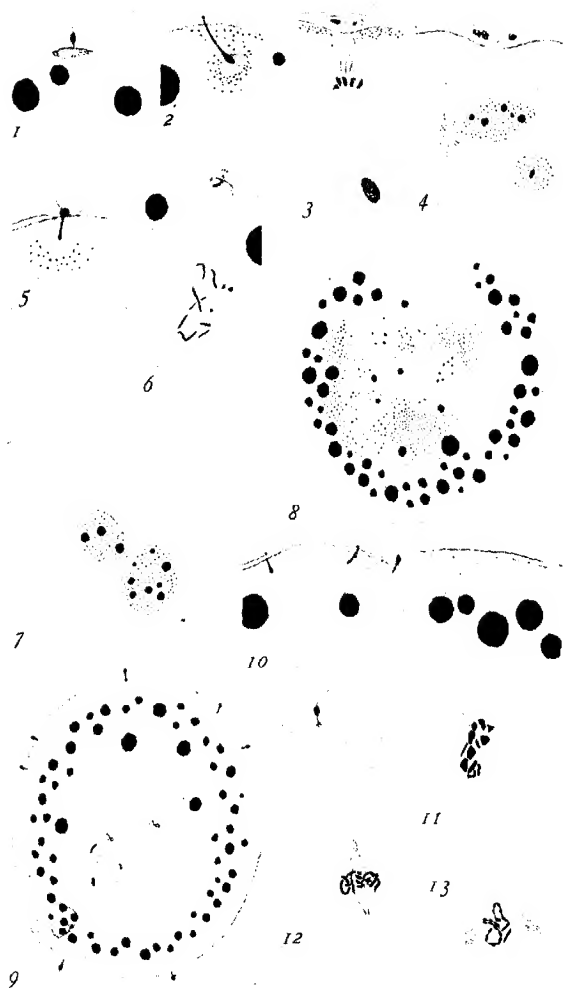
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All the drawings were made with a camera, with a 2 mm. Zeiss apochromatic objective, and a No. 6 compensating ocular, unless otherwise stated.

## PLATE 1

### EXPLANATION OF FIGURES

- 1 to 4 Normal Fertilization.
- 1 Attachment of the sperm; 55 minutes after insemination.
- 2 Penetration of the sperm head; 60 minutes after insemination.
- 3 Telophase of the second maturation division, and approach of the sperm nucleus; 65 minutes after insemination.
- 4 Just before the fusion of the germ nuclei; 90 minutes after insemination.
- 5 to 9 Fertilization of normal eggs by radiated sperm.
- 5 Penetration of the sperm head, showing absence of the fertilization cone; 60 minutes after insemination.
- 6 Development of the egg nucleus, and failure of the sperm nucleus to enter; 115 minutes after insemination.
- 7 Gradual disappearance of the sperm aster; 105 minutes after insemination.
- 8 Total disappearance of the sperm aster. The sperm nucleus has divided into many vesicles; 115 minutes after insemination.
- 9 to 29 Fertilization of the radiated eggs by normal sperm.
- 9 Polyspermy. This egg has failed to extrude the alveolar layer. 2 mm. apochr. and No. 0 eyepiece.
- 10 Detail of the same; 70 minutes after insemination.
- 11 Formation after the first maturation figure without astral system.
- 12 Same, with spindle but no asters; 45 minutes after insemination.
- 13 Same. The chromatin is segmented, but is splitting longitudinally 45; minutes after insemination.



## PLATE 2

### EXPLANATION OF FIGURES

- 14 First maturation division occurring at a point where the alveolar layer has been extruded; 60 minutes after insemination.
- 15 Entrance of the sperm, showing the non-staining vacuole.
- 16 Tripolar spindle of the first maturation; 55 minutes after insemination.
- 17 First maturation spindle, showing an abnormal distribution of the chromosomes; 60 minutes after insemination.
- 18 The second maturation. The first polar body has not been extruded; 80 minutes after insemination.
- 19 The same; two sperms are entering in one, the aster has appeared before the revolution of the cone. 2 mm.  $\times$  No. 0.
- 20 The cleavage nucleus. The chromatin is passing out through the nuclear wall; 120 minutes after insemination.
- 21 Polyspermy; 70 minutes after insemination.
- 22 Division of the protoplasm without a distribution of the chromatin. 2 mm. apochr.  $\times$  No. 0 eyepiece.
- 23 The same; the same magnification.
- 24 to 30 Normally fertilized eggs radiated.
- 24 Approach of the germ nuclei; 105 minutes after insemination.
- 25 The nuclei have migrated to opposite sides of the egg; 105 minutes after insemination.

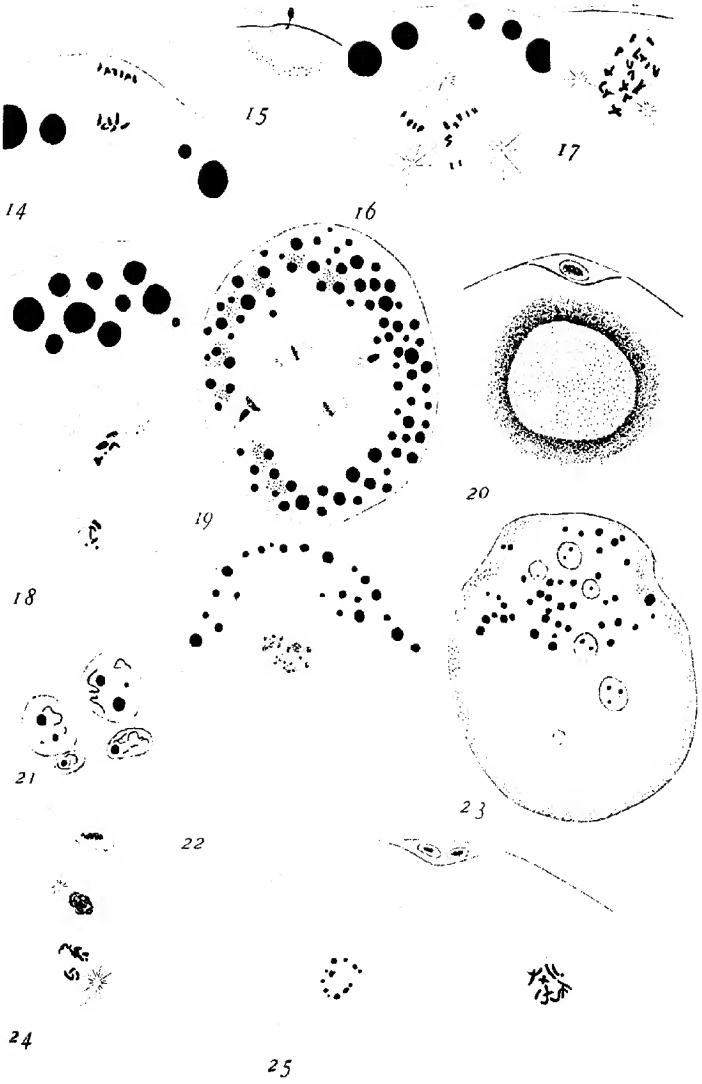
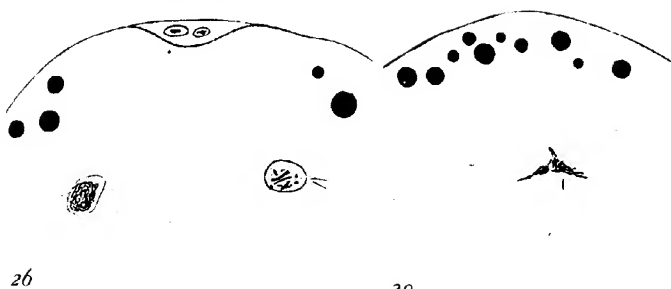


PLATE 3

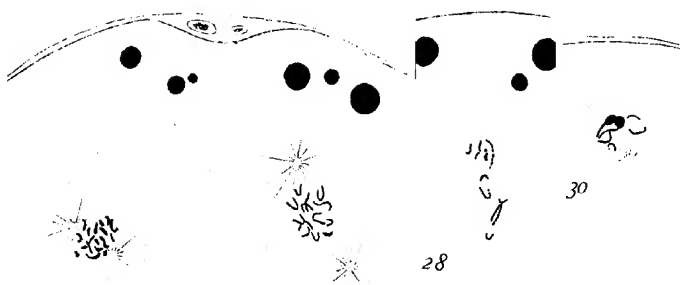
EXPLANATION OF FIGURES

- 26 The same.
- 27 Each germ nucleus is dividing by itself; 115 minutes after insemination.
- 28 Disintegration of the germ nuclei; 115 minutes after insemination.
- 29 Tripolar spindle; 120 minutes after insemination.
- 30 Degeneration of the germ nuclei; 120 minutes after insemination.
- 31 to 34 Eggs radiated both before and after fertilization.
- 31 Abnormal polar body formation; 60 minutes after insemination.
- 32 The same.
- 33 The egg showing the phenomenon of budding.
- 34 The same; both 120 minutes after insemination.



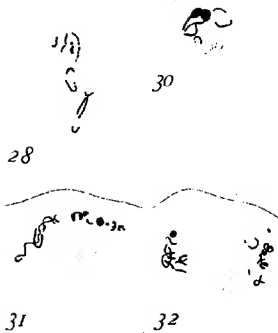
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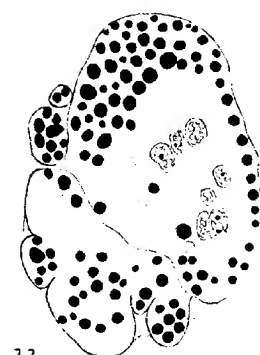
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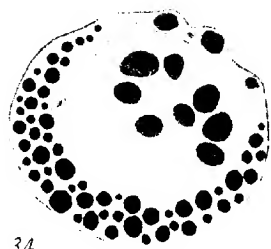
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# A FURTHER STUDY OF SIZE INHERITANCE IN DUCKS WITH OBSERVATIONS ON THE SEX RATIO OF HYBRID BIRDS

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## SEVEN CHARTS

In a previous number of this journal the writer reported the result of a cross between two races of ducks differing in size. The number of offspring produced was small, but tended to show a marked increase of variability in size in  $F_2$ .

In the breeding season of 1912 more data were obtained from the same stocks, 57  $F_1$  and 31  $F_2$  ducks being raised to maturity. Growth curves were made for all these ducks from an early age until such time as they were considered adult, or nearly so. The average length of this period is 142 days. The growth curves give a good check on the adult or autumn weight of the individual, and enable one to assign the proper weight figure to each bird with very fair precision. The ducks were weighed at intervals of one week to two weeks.

The curves will be considered later on, together with the growth rate of a race of pure wild mallards reared in 1912.

As a further test of the parent stocks, which it may be recalled consisted respectively of Rouen ducks and domesticated mallards of a particular strain, 9 pure Rouen and 20 mallards were reared in 1912. The weight of these animals at five months of age is recorded in table 8.

To produce another lot of  $F_1$  ducks, one of the original Rouen males was mated with three female mallards taken at random from among the same birds used in the previous work. For the  $F_2$  generation, the same three  $F_1$  females as had been used the previous year, Nos. 83, 101, and 106, were mated with  $F_1$  male

TABLE 1  
*Summary of entire series of observations on weight in Rouen and mallard ducks, and in hybrids between these races*

	GROUP	AGE	NUMBER OF MALES	NUMBER OF FEMALES	MEAN WEIGHT OF MALES IN GRAMS	MEAN WEIGHT OF FEMALES IN GRAMS	STANDARD DEVIATION OF MALES	STANDARD DEVIATION OF FEMALES	C. V. MALES	C. V. FEMALES
Rouen.....	original stock	1½ years	2	4	2790	2585				
	2d gen.	5 mos.	2	5	2535	2396				
	3d gen.	5 mos.	6	9	2250	2149				
	4th gen.	5 mos.	4	5	2610	2330				
Mallard.....	2, 3 and 4 combined	5 mos.	12	19	2417	2261	225	171	9.3	7.56
	original stock	5 mos.	about 30	about 35						
	2d gen.	5 mos.	59	43	1068	928	119	76	11.14	8.18
	3d gen.	5 mos.	7	13	1148	1074	112	148	9.76	13.6
1910 and 1911 hybrids.....	F <sub>1</sub> gen.	5 mos.	10	3	1605	1587	97	54	5.82	3.43
	F <sub>2</sub> gen.	134 to 151 days	17	16	1781	1684	224	181	12.57	11.07
	F <sub>1</sub> gen.	134 to 160 days	36	21	1660	1479	173	134	10.42	10.5
	F <sub>2</sub> gen.	133 to 159 days	17	14	1700	1546	306	162	18.00	10.5
Totals of hybrids.....	F <sub>1</sub> gen.	5 mos.	46	24	1661	1492	158	152	9.5	10.1
	F <sub>2</sub> gen.	5 mos.	34	30	1740	1592	269	174	15.4	10.9

TABLE 2  
*Summary of measurements of 1912 ducks in centimeters*

			BILL	TARSUS	NECK	TOTAL LENGTH
Males	F <sub>1</sub> .....	mean.....	5.5	5.24	35.6	68.2
		S.D.....	0.27	0.31	1.69	2.53
		C.V.....	4.90	5.91	4.74	3.71
	F <sub>2</sub> .....	mean.....	5.5	5.12	35.5	68.0
		S.D.....	0.29	0.29	2.22	3.67
		C.V.....	5.27	5.66	6.26	5.39
Females	F <sub>1</sub> .....	mean.....	5.21	5.0	32.9	61.2
		S.D.....	0.23	0.19	1.31	3.54
		C.V.....	4.22	3.80	3.98	5.78
	F <sub>2</sub> .....	mean.....	5.35	5.0	32.3	63.1
		S.D.....	0.24	0.19	1.98	2.51
		C.V.....	4.48	3.80	6.13	3.97

103, for the first three clutches of eggs, with F<sub>1</sub> male 105 for the last two clutches (see former paper). Female 83 died after the first few eggs had been taken from the pen, so that nearly the entire F<sub>2</sub> lot was raised from only two mothers. Is it thus evident that as regards the number of the parent individuals in each lot there is fully as great a chance for variability among the F<sub>1</sub> ducks as among the F<sub>2</sub> ducks. Both generations ran together in the rearing yard and in the maturing yard in exactly the same way as in 1910-1911. The weights of the birds reared in 1912 are given in tables 4 to 7. In table 1 are given the means and certain other statistical constants for each lot of birds together with the results obtained in the two previous years, thus summarizing the entire experiment.

The standard deviation in weight of the 1912 birds is greater than that of the 1910 and 1911 birds. The mean weight of the 1912 birds is somewhat less than that of the 1910-1911 birds, especially the F<sub>2</sub> birds.

It is plain that, in spite of the greater variability of the 1912 F<sub>1</sub> birds, induced no doubt mostly by their greater number (57 animals instead of 13), there is still a small excess of variability of the F<sub>2</sub> over that of the F<sub>1</sub> generation (at least among the males).

Finally, the combination of the three years' work gives a very satisfactory basis for comparison, the F<sub>1</sub> lot comprising 70 animals

and the  $F_2$  lot 64. The results for 1912 are not, however, so striking as those for 1911, but point in the same direction—to a partial segregation of multiple size factors, or to a modification of the gametes brought about by their association in  $F_1$  zygotes. The increased variability in  $F_2$  is far greater in the male sex than in the female.

As a further index of size differences, external measurements were taken on all the adult 1912 ducks. These were as follows:

Bill: exposed culmen.

Tarsus: the tibio-tarsal measurement as used by the ornithologists.

Neck: from the anterior point of the breast bone to the tip of the bill. The front end of the breast bone is a point which can readily be felt. This measurement is easier after rigor mortis has passed.

Length: tip of bill to end of tail feathers.

A summary of these measurements is given in table 2, for each sex and generation. Among the eight pairs of coefficients of variability obtained the  $F_2$  coefficient is greater than the  $F_1$  coefficient in five cases, the same in one case, and less than the  $F_1$  in two cases. Accordingly greater variability of the  $F_2$  birds is not very clearly shown in the measurements taken.

#### GROWTH OF DUCKS

In a recent paper Goldschmidt gives the result of some size work in ducks. He does not deal with adult weights, but with a coefficient of growth determined by dividing the weight at ten weeks by the first or hatching weight. In this way he investigated the early growth rate of several breeds. He obtained growth figures for one lot of  $F_2$  Peking female mated with wild mallard male, and records a great divergence, which he attributes partly to segregation. It is to be remarked that Goldschmidt's wild mallard race reached a weight of 1470 grams, which would suggest that it might have been contaminated, for this weight is very high. The average for my original stock of wild mallards is 1073 grams for the males, and 922 grams for the females, while the autumn, or five month's weights for the offspring were 949 and

892 grams respectively. The difficulty of obtaining pure wild mallards in parts of Europe is well brought out by Rogeron in his work "Les canards."

Goldschmidt lays stress on the importance of environmental factors in the growth rate of ducks. To show the influence of earliness or lateness of hatch on body weight, I have prepared table 3. In this table each of the four groups of 1912 ducks (both sexes and both generations) were split into early and late hatches, and the average age and average adult weight found for each of the eight groups thus obtained. The result is very striking, and shows the advantage of lateness over earliness in every one of the four cases.

The growth curves show the same phenomenon. For example in chart 4, curve  $F_1\varphi 1$  represents the weights of early hatched birds and shows the greatest weight at 60 days of age but a very low maturity weight at 105 days. On the other hand, curve  $F_2\varphi 5$  (late hatched birds) shows a smaller weight at 60 days of age but much greater weight at maturity.

The early hatched birds tend to mature quicker and remain smaller. This result only emphasizes the importance of outside conditions as pointed out by Goldschmidt.

In charts 2 to 5 growth curves are given for both sexes and both generations of the cross-bred birds. Each curve represents the average growth of one sex of each clutch of eggs. At the point marked *P*, at 50 to 70 days of age, there is always a depression which corresponds with the removal of the ducklings from the rearing yard to the maturing yard, where the free access to water and greater space tended for a short time to keep their weight down.

There is a slight tendency to a depression at the time of moult from the first juvenile to the first adult plumage, which takes place between the ages of 110 and 140 days. In all cases the birds were in full plumage when killed, and the completion of the full plumage is a very good criterion of the time when the adult or autumn weight has been attained. Increase in weight beyond this point seems to be due mostly to an increase of fat, and is generally much more marked among the females than among the

TABLE 3  
*Relation between size of 1912 ducks and early or late hatching*

F <sub>1</sub> GENERATION		F <sub>2</sub> GENERATION	
19 early males.....	approximate date of hatching, May 27 approximate age when killed, 140 days average weight in grams, 1532 grams approximate date of hatching, June 20	11 early hatched males.....	approximate date of hatching, May 27 approximate age when killed, 140 days average weight in grams, 1609 grams approximate date of hatching, June 18
17 late males.....	approximate age when killed, 140 days average weight in grams, 1743 grams approximate date of hatching, May 29	6 late hatched males.	approximate age when killed, 145 days average weight in grams, 1873 grams approximate date of hatching, May 24
11 early females...	approximate age when killed, 145 days average weight in grams, 1404 grams approximate date of hatching, June 18	7 early hatched females.....	approximate age when killed, 148 days average weight in grams, 1405 grams approximate date of hatching, June 19
10 late females.....	approximate age when killed, 140 days average weight in grams, 1562 grams	7 late hatched males.	approximate age when killed, 146 days average weight in grams, 1687 grams

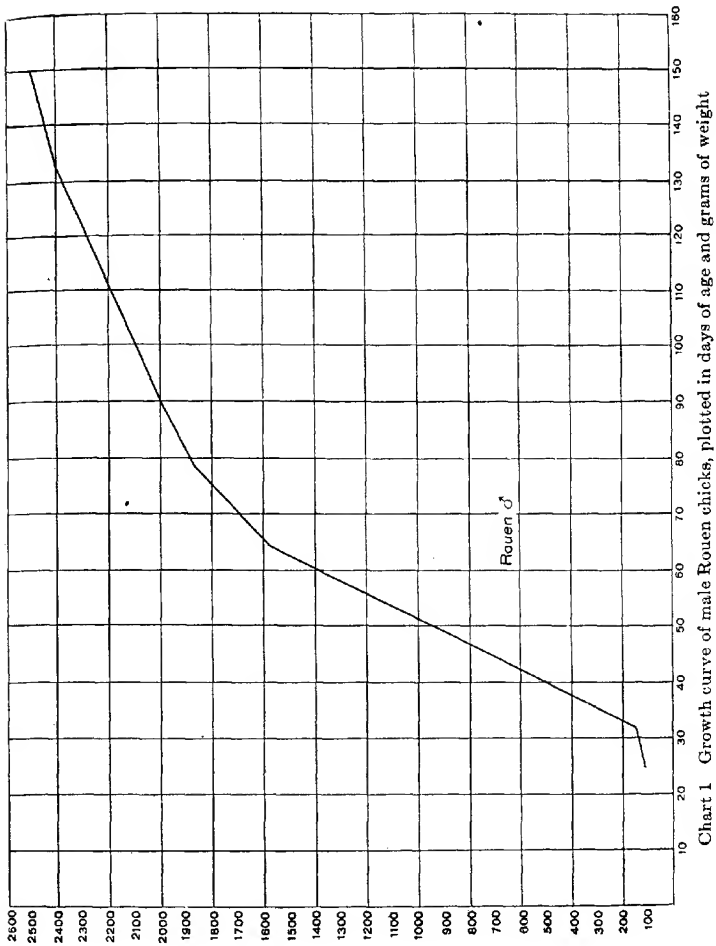


Chart 1 Growth curve of male Rouen chicks, plotted in days of age and grams of weight



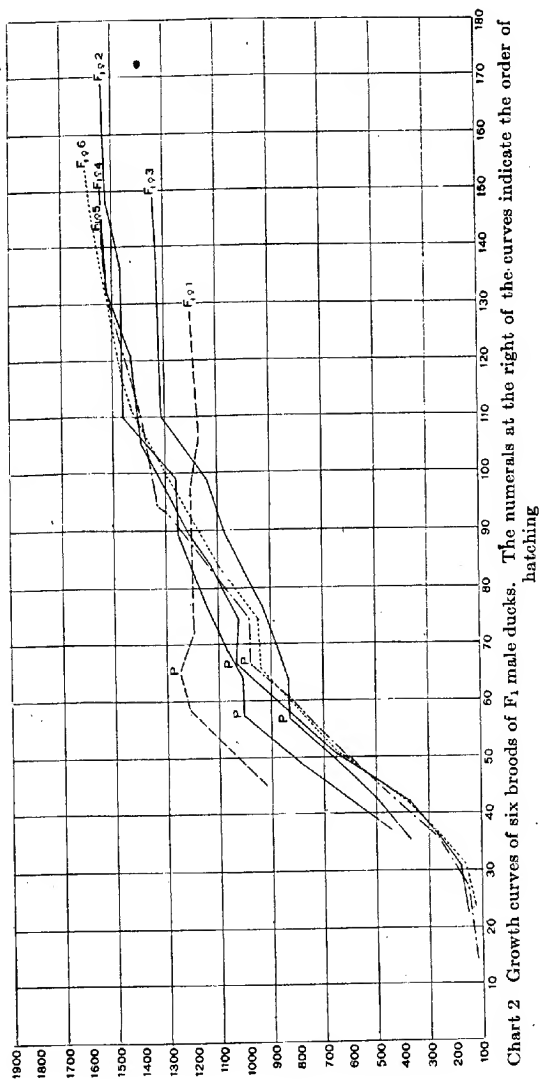


Chart 2 Growth curves of six broods of  $F_1$  male ducks. The numerals at the right of the curves indicate the order of hatching

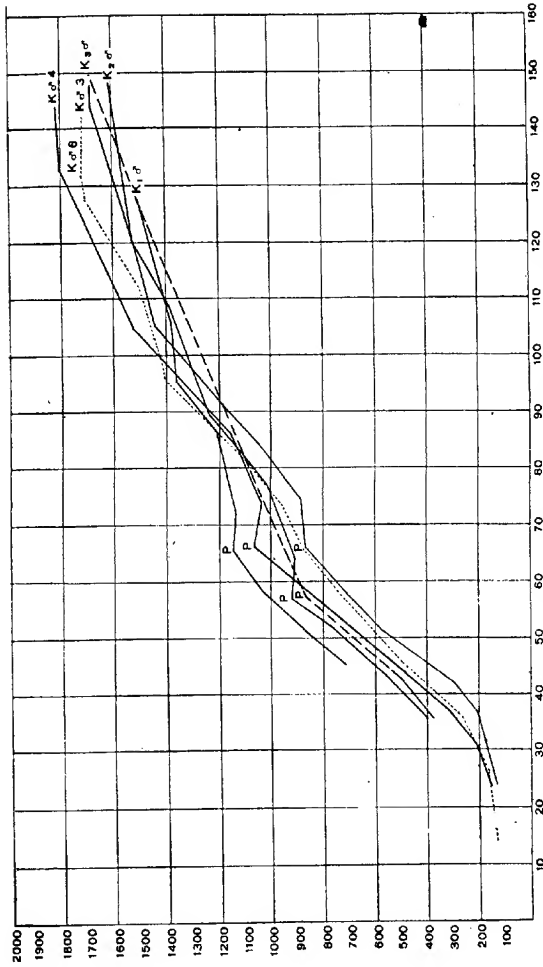
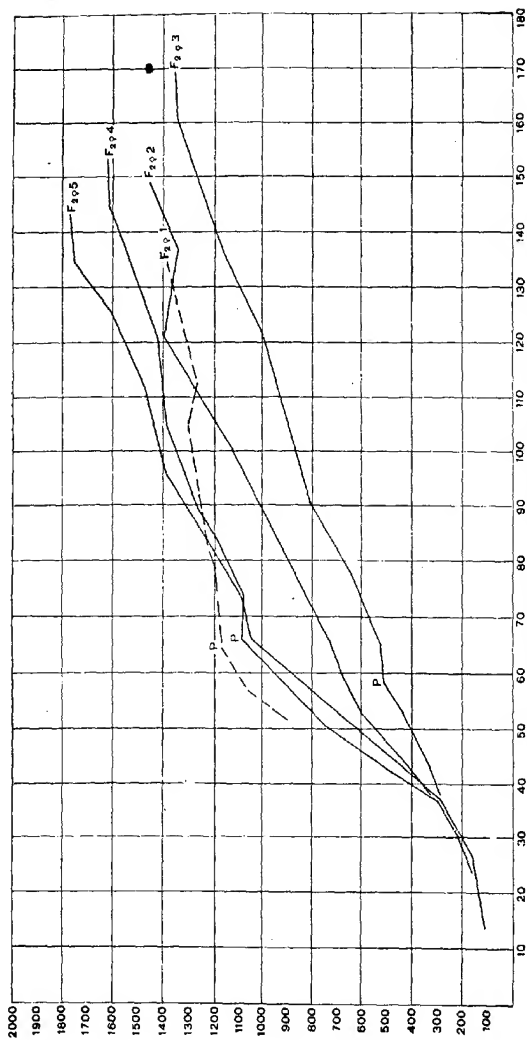


Chart 3 Growth curves of six broods of F<sub>1</sub> female ducks

Chart 4 Growth curves of five broods of female  $F_2$  ducks

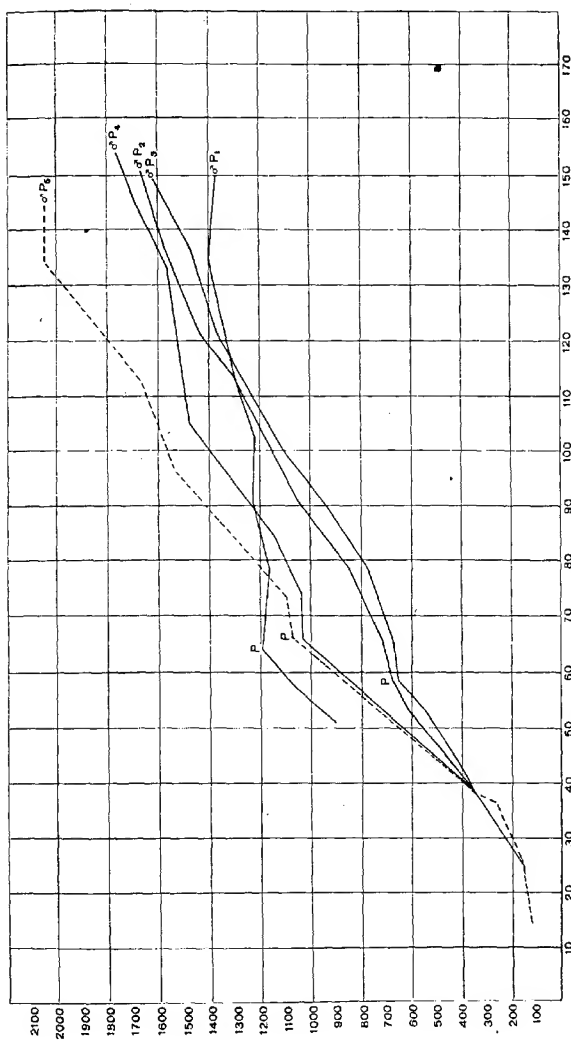


Chart 5 Growth curves of five broods of  $F_2$  male ducks

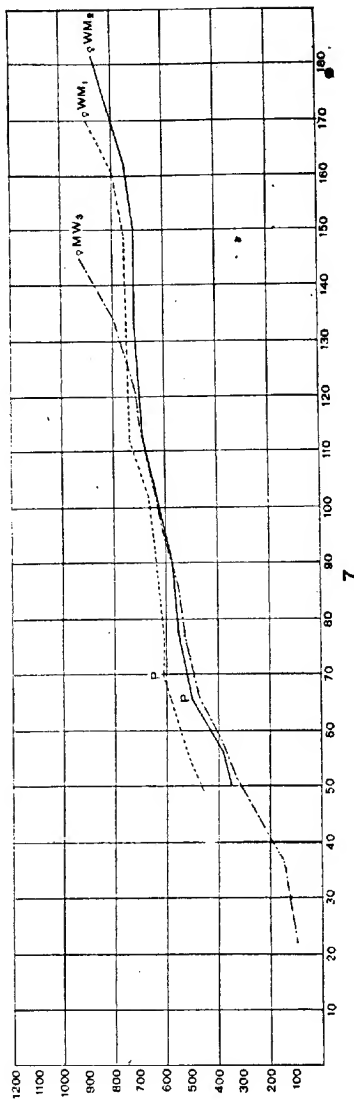
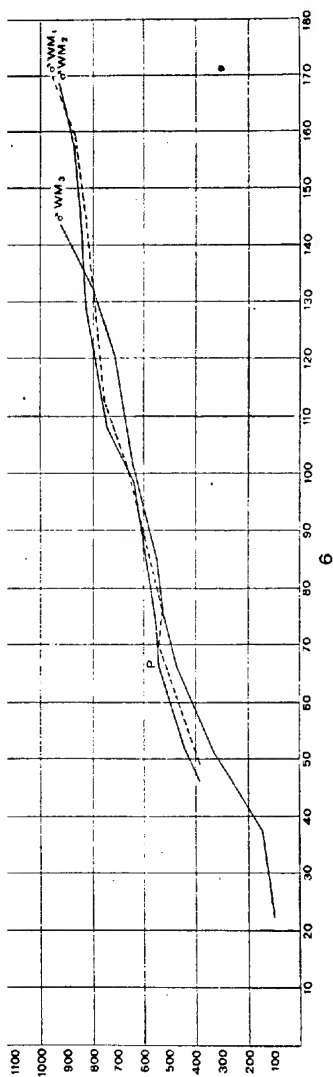


Chart 6. Growth curves of three broods of pure wild male mallards

Chart 7. Growth curves of three broods of pure wild female mallards

males. The small figures at the end of the curves refer to the sequence in date of hatching time of the different clutches of eggs.

The curve for the Rouen ducks shows the remarkable growth of this race between the ages of 30 and 70 days. The curves for the small race are not available, but I have added two curves for 26 individuals of a pure wild race of mallards in charts 6 and 7. These birds are slightly smaller and much more uniform in size than the 'English' mallards. They show a standard deviation of 43.5 for the males and 23.93 for the females, and a coefficient of variability of 4.5 for the males and 2.6 for the females. The males, as among the other races, show a much greater tendency to vary. This fact may be a universal one for ducks; it is in agreement with the more uniform c. v. for the females in both  $F_1$  and  $F_2$  generations.

#### INHERITANCE OF COLOR IN ROUENS

This is mentioned because Goldschmidt (p. 189) expresses some doubt as to the homozygous nature of the color of the Rouens. In my strain at least there is no question about this. In crosses with mallards, 46  $F_1$  males were all true to the mallard type of pattern, while 34  $F_2$  males gave only two birds which were slightly 'off color.' These two birds had large white collars with some white on the breasts and primaries. Goodale showed in crosses between Pekins and Rouens that a very complicated assemblage of color types resulted in the first hybrids. He attributes this to heterozygosis among the Pekin males, which view would appear to be the correct one.

#### THE SEX RATIO OF $F_1$ SIZE DUCKS

Owing to the present general interest in disturbed sex ratios of hybrid animals it is worth while to call attention to the results obtained in ducks (table 1). In both seasons there was a great preponderance of males among the  $F_1$  offspring though among the  $F_2$  birds, where the parents were similar in size, the sexes are nearly equal. Adding the results for both years together, we obtain

the following proportions of the sexes: In  $F_1$ , males 46, females 24; in  $F_2$ , males 34, females 30.

In the first case is found practically a two to one ratio, which, in view of the large number of observations, is significant. Guyer called attention to an excess of males in various hybrid birds, especially when the parents were from widely separated species or different genera. His results are amply confirmed in a recent paper by Geoffrey Smith and Mrs. Haig-Thomas on pheasants. An excess of males is apparent in fertile hybrids, but seems to be greater in sterile hybrids. I myself have found that in hybrids between Reeves and *Torquatus* pheasants, Jungle fowl crossed with pheasants, and Swinhoe pheasants crossed with Silver, a great excess of males occurs.

It is therefore very interesting to find the same result obtaining in a cross between two domestic races of ducks, very dissimilar in size, but derived from the same wild species, and producing fertile offspring when intercrossed. It is possible to explain the inequality of the sexes as due to a selective death rate on the part of the two sexes, a larger proportion of male zygotes surviving, but the supposed selective death rate, if it occurs at all, must be effective very early in embryonic life, for out of 76 eggs set in 1912, 60 hatched, and 57 were reared to maturity, producing 36 males and 21 females. Most of the eggs, which failed to produce young were sterile or at least discarded as such after ten days incubation (exact number not noted) so that a selective fertilization seems to be a more probable explanation in this case, but an early embryonic selective mortality cannot be ruled out.

In conclusion I wish to express my thanks to Professor Castle for help in working out details.

#### SUMMARY

1. The high coefficient of variability of both the parent races, especially the mallards, may perhaps increase the variability in  $F_1$ . Possibly the mallard race may contain an admixture of the small, so called 'toy' race, which would account for some of the small individuals which occasionally appear. In spite of this, however, the male ducks show a very considerably increased variability

of weight in  $F_2$  over their variability in  $F_1$  and the females show a very small increase. The numbers are now thought large enough to have a significance.

2. If a stock like the pure wild race mentioned above, could be used as a small parent, it is very likely that a much more striking segregation of size might be observed.

3. The present experiment does not throw any further light on the theoretical side of the question except to diminish the possibility of the existence of any large and clear cut size units in birds, which would result in an easily recognized numerical ratio.

4. The male sex is much more variable in size than the female.

5. The Rouen race used in this work was homozygous for color (mallard color) when crossed with a mallard race.

6. The growth curves show that a very satisfactory age for studying size of ducks is 140 to 150 days, at the assumption of the first adult plumage, but also point to a marked effect of earliness and lateness of hatch on the rate of growth and ultimate size. This difference probably varies greatly from year to year and cannot be regarded as uniform.

7. A disturbed sex ratio occurs among the  $F_1$  hybrids, a result apparently of the difference in size of the parents, for it is not seen in  $F_2$ . This ratio has resulted in the preponderance of males over females in the proportions of nearly two to one.

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TABLE 4  
*Weights and measurements of  $F_1$  male ducks reared in 1912*

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			grams	cm.	cm.	cm.	cm.
305	May 23	Sept. 28	1740	5.9	5.5	35.5	68.0
306	May 23	Sept. 28	1290	5.3	5.1	31.5	61.2
307	May 23	Sept. 28	1470	5.6	5.3	35.5	67.3
308	May 23	Sept. 28	1830	5.8	5.5	34.3	67.1
310	May 23	Sept. 28	1100	4.9	4.6	28.7	58.5
312	May 23	Sept. 26	1760	6.1	6.5	38.5	72.0
313	May 30	Nov. 6	1690	5.9	4.5	37.0	70.5
314	May 30	Nov. 6	1700	5.5	5.3	37.1	69.9
318	May 30	Nov. 6	1640	5.6	5.3	36.0	69.8
319	May 30	Oct. 14	1600	5.3	5.0	35.8	68.2
320	May 30	Oct. 26	1720	5.4	5.4	36.3	69.0
321	May 30	Oct. 26	1500	5.9	5.4	36.0	68.6
323	May 30	Oct. 26	1590	5.6	5.3	35.0	67.0
325	May 30	Oct. 14	1500	5.3	5.15	35.0	66.5
326	May 30	Oct. 14	1600	5.5	5.15	36.8	69.0
344	May 30	Oct. 14	1600	5.8	5.2	36.8	70.0
346	May 30	Oct. 26	1460	5.2	4.9	35.7	66.8
347	May 30	Nov. 6	1770	5.4	5.1	35.5	68.5
348	May 30	Nov. 6	1550	5.2	5.1	35.1	66.2
351	May 30	Nov. 6	1680	5.4	5.3	36.2	69.0
354	June 15	Oct. 26	1655	5.3	5.3	36.1	69.2
358	June 15	Nov. 6	1860	6.1	5.5	35.0	69.0
360	June 15	Nov. 6	1890	5.8	5.2	37.2	70.4
361	June 15	Oct. 26	1570	5.4	5.3	37.6	70.5
365	June 15	Oct. 26	2130	5.6	5.3	36.5	70.8
370	June 15	Nov. 15	1700	5.8	5.4	33.6	68.8
371	June 15	Nov. 6	1560	5.4	5.0	37.8	69.7
373	June 15	Nov. 6	1750	5.4	5.2	35.0	68.0
375	June 15	Nov. 15	1700	5.4	5.2	35.5	68.6
378	June 25	Nov. 15	1750	5.5	5.2	34.5	68.3
379	June 25	Nov. 15	1630	5.9	5.4	36.2	69.8
381	June 25	Nov. 15	1800	5.7	5.1	35.4	68.8
383	June 25	Oct. 14	1700	5.2	5.7	35.5	67.0
385	June 25	Nov. 15	1800	5.8	5.5	36.2	69.9
387	June 25	Nov. 6	1700	5.3	5.1	35.0	67.5
390	June 25	Nov. 6	1770	5.9	5.2	36.4	69.7

TABLE 5  
Weights and measurements of  $F_1$  female ducks reared in 1912

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			grams	cm.	cm.	cm.	cm.
309	May 23	Sept. 28	1200	5.4	5.0	33.0	62.9
315	May 30	Oct. 14	1500	5.1	5.0	31.6	64.0
317	May 30	Oct. 26	1560	5.1	5.2	33.8	65.0
324	May 30	Oct. 26	1385	5.3	5.1	32.2	63.9
327	May 30	Nov. 6	1580	5.2	4.9	32.3	63.4
330	May 30	Nov. 6	1420	5.6	5.0	34.2	66.0
335	May 30	Nov. 26	1450	5.3	4.8	33.1	63.8
338	May 30	Nov. 26	1230	4.9	4.7	31.5	61.8
340	May 30	Oct. 14	1500	5.15	4.9	34.0	62.0
342	May 30	Oct. 14	1330	5.0	4.8	32.0	63.0
343	May 30	Oct. 14	1290	4.8	4.7	32.1	63.0
350	June 15	Oct. 14	1390	5.1	4.9	32.4	63.6
359	June 15	Oct. 15	1700	5.6	5.4	33.5	66.0
364	June 15	Oct. 15	1600	5.3	5.1	32.5	63.8
367	June 15	Oct. 26	1530	4.9	4.9	30.8	62.3
369	June 15	Oct. 26	1380	5.3	5.0	34.2	64.4
372	June 15	Nov. 6	1900	5.7	5.5	37.2	70.7
374	June 15	Nov. 6	1540	5.2	5.1	32.5	64.0
386	June 25	Nov. 6	1450	5.1	5.0	33.6	65.0
388	June 25	Oct. 26	1610	5.3	5.2	32.7	65.7
391	June 25	Nov. 15	1520	5.1	5.1	32.2	64.8

TABLE 6  
Weights and measurements of  $F_2$  male ducks reared in 1912

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			grams	cm.	cm.	cm.	cm.
247	May 17	Oct. 14	1330	5.25	4.7	35.0	66.0
288	May 17	Sept. 28	1360	5.85	5.1	35.5	66.3
311	May 30	Oct. 26	2180	5.6	5.0	38.0	72.3
316	May 30	Oct. 13	1700	5.65	5.4	37.0	69.0
328	May 30	Oct. 26	1680	5.4	5.2	38.0	69.8
329	May 30	Oct. 13	1490	5.1	5.1	34.6	65.0
331	May 30	Nov. 6	1645	5.5	5.2	36.0	69.0
332	May 30	Nov. 15	1945	6.0	5.6	38.0	72.0
336	May 30	Oct. 13	1670	5.5	4.9	36.0	67.0
341	May 30	Oct. 26	1100	4.8	4.5	29.7	58.4
349	May 30	Nov. 15	1600	5.8	4.5	36.7	69.5
352	June 15	Nov. 15	1480	5.2	5.2	33.0	65.0
356	June 15	Nov. 6	1870	5.5	5.5	35.5	69.0
362	June 15	Oct. 26	1890	5.4	5.1	35.3	69.2
368	June 15	Nov. 15	1850	5.2	5.2	32.5	66.5
377	June 25	Nov. 15	1750	5.6	5.3	34.8	68.0
389	June 25	Nov. 15	2400	5.8	5.7	38.0	75.0

TABLE 7

*Weights and measurements of F<sub>2</sub> female ducks reared in 1912*

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			<i>grams</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>
283	May 17	Sept.28	1330	4.7	4.9	29.4	58.0
289	May 17	Sept.28	1390	5.1	4.7	29.6	59.5
296	May 17	Sept.28	1450	5.25	5.1	32.0	62.0
333	May 30	Oct.26	1520	5.7	4.9	34.4	66.0
337	May 30	Nov.15	1430	5.5	5.2	34.0	65.0
339	May 30	Oct.26	1415	5.6	5.0	32.5	64.0
345	May 30	Nov.15	1300	5.1	4.8	30.0	60.0
353	June 15	Nov.15	1600	5.4	4.9	30.8	62.5
357	June 15	Oct.26	1600	5.8	5.1	34.2	66.4
363	June 15	Nov.6	1600	5.3	4.9	31.0	63.8
366	June 15	Oct.15	1640	5.4	5.3	31.2	65.0
376	June 25	Oct.15	1800	5.4	5.3	30.0	62.5
380	June 25	Oct.15	1770	5.0	5.0	29.9	62.8
382	June 25	Oct.15	1800	5.6	5.4	33.8	66.5

TABLE 8

*Weights in grams at five months of age of Rouen and mallard ducks reared in 1912*

ROUEN MALES	ROUEN FEMALES	MALLARD MALES	MALLARD FEMALES
2440	2290	950	830
2570	2320	1080	940
2680	2340	1120	970
2750	2340	1150	980
	2360	1150	1020
		1290	1045
		1300	1060
			1100
			1110
			1160
			1200
			1240
			1310

EXPERIMENTAL EVIDENCE CONCERNING THE  
DETERMINATION OF POSTURE OF THE  
MEMBRANOUS LABYRINTH IN  
AMPHIBIAN EMBRYOS

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THIRTY-EIGHT FIGURES

The purpose of the present paper is to report a series of experiments that substantiate the conclusion that the posture of the membranous labyrinth and the position of its canals is determined by some force or influence that interacts between the labyrinth and its environment. The exact nature of this control or influence has not yet been determined, but from the experiments that are to be described it is evident that it possesses a force capable of producing a complete rotation of an embryonic vesicle that has been displaced in a reversed position.

The existence of a postural influence of this kind introduces a new factor in organogenesis. It means that organs do not develop inertly in the position that they happen to find themselves; on the contrary, there is a certain amount of adjustment of position through forces interacting between them. According to the conception of what we might call the *theory of passive organ development*, all organs develop passively in the position in which they are located at the outset. Perfect form according to this theory is eventually obtained because originally the anlagen of the various organs are so perfectly placed, and all the stresses so carefully calculated, that the subsequent increase in size, and mutual pressure against each other, produce a final normal disposition of all of them. In contrast to this we may now speak of *self-placement of organs* or *individuality of organ development*, according to

which there is, in addition to the passive pressures of adjacent structures, a motive element, the *force of placement*, which helps to maintain, and corrects if necessary, the relative position of an individual organ. This *force of placement* may be defined as the product of the combined motive forces interacting between an organ and its environment. We are already familiar with the striking phenomenon of muscle migration and the movement of nerve-ganglion masses. We can now add to this the potential movement and adjustment of entire organs during their course of development, and it is not necessary to add that this *autostatic tendency* is doubtless much more pronounced in some organs than in others.

The writer's attention was first directed to the possibility of experimentally altering the position of the developing labyrinth, during some investigations on equilibration in amphibian larvae (Streeter '06). In these experiments it was found that the cells constituting the ear vesicle are specialised very early, and though transplanted to an abnormal environment they continue to differentiate themselves in the usual way into a recognizable labyrinth. Lewis ('07) showed in a subsequent paper that this was true while they were still in the stage of an uninvaginated plate, quite in contrast to the surrounding cells forming the cartilaginous capsule, which Lewis clearly proved were not predetermined in this way.

In a later paper (Streeter '07) additional evidence was given of the high degree of developmental independence possessed by the early labyrinth cells. It was shown that even fragments of the vesicle may develop independently of the rest of the vesicle, and any individual part, for example the endolymphatic appendage, may be quite normal in cases where the remainder of the labyrinth is very abnormal. It was also shown (Streeter '09) that when two primitive vesicles are crowded together into the same pocket they do not fuse and form together one large labyrinth, but remain as two distinct labyrinths. Moreover, it was shown that this developmental independence of the vesicles extends to the difference existing between a right and left-sided organ. The dextral or sinistral character, or laterality of the ear

vesicle, is not controlled by the environment but is determined by the intrinsic character of its own constituent cells. A left-sided ear vesicle when transplanted to the right side develops into a labyrinth having all the characteristics of a left-sided organ; the anterior canal is formed on the caudal border of the labyrinth, the posterior canal on the oral border, and the lagena, which normally is directed caudalward, is found extending forward toward the eye.

It was found, however, that the ear vesicle, though capable of this marked power of self-differentiation, apparently was not in all respects independent of the surrounding structures. The posture of the developed labyrinth, the situation of its canals and various chambers, seemed to be controlled by the environment. Deliberate rotation of the ear vesicle into abnormal positions and even transplantation to the opposite side of the body, always resulted in a labyrinth possessing a normal attitude with reference to the brain, ganglion masses, and the surface of the body. In all of seventeen experiments performed for this purpose the results were positive, and the writer consequently came to the conclusion that there is some influence interacting between the ear vesicle and its environment that constitutes the chief factor in the determination of its placement.

At the same time an experienced foreign investigator had been working independently upon a similar problem (Spemann '06 a and '06 b). His experiments consisted in removing the ear vesicle in young *Rana esculenta* larvae and then replacing it in an inverted position for the purpose of studying the consequent abnormal body movements and their correlation with the anatomical results of the operation. He also studied the influence of the epithelial labyrinth upon the surrounding connective tissue, and its relation to the formation of the cartilaginous and bony labyrinth. Concerning this latter problem he did not come to a definite conclusion. The former problem, however, gave positive results. He found that the inverted vesicle continued in its development and formed a more or less complete labyrinth, but, unlike my specimens, the orientation of the developed labyrinth corresponded to the operative displacement of the vesicle. There had been no subsequent readjustment of position. After the appearance of

my papers (Streeter '06 and '07) in which I reported the experiments in which, after various displacements, the labyrinths all recovered their normal postural relations, Spemann ('10) made further experiments and reported more at length with regard to postural development. His results confirmed his earlier observations, and his paper contained illustrations showing sections of larvae of different ages in which the inverted ear vesicle has remained inverted though otherwise practically normal. Out of twelve operations nine labyrinths remained inverted, two recovered partly, and one recovered perfectly the normal position. Regarding the latter specimen he surmised that it had not been sufficiently turned, and had slipped back into its normal position. The results, therefore, as obtained by Spemann are directly contradictory to those obtained by me. On account of the great embryological significance of the question of environmental postural control, further experiments were plainly warranted both for the purpose of testing my own previous results and if possible for finding some explanation of the discrepancy existing between the observations of Spemann and myself. From the study of these new experiments and from an examination of the accompanying photographs it will be plainly apparent that they confirm my previous results. Before entering into a further discussion of them we may proceed with a description of the details of the experiments themselves.

#### METHOD OF OPERATION

Obviously the conditions of the operation should be planned so that the environmental control would be put to an extreme test. In most of my previous experiments, and in all of Spemann's, the vesicle operated upon, after being taken out and rotated one way or another, was replaced in its own pocket. Recovery of the original posture after such an operation might be explained by the possibility of the vesicle not having been completely detached; some small strand of tissue might suffice to draw it back into the original position. Also it is conceivable that, since the vesicle and any adherent mesodermal fragment exactly fitted the pocket

from which they were taken, they might on that account slip back into their original position. Both of these objections were avoided by transplanting a vesicle from one specimen into the emptied ear pocket of another specimen. This certainly involved complete detachment, and it is obvious that the transplanted ear vesicle would not exactly fit in a pocket in which it did not originally belong. All of the following experiments were done in that way.

The experiments were carried out on larvae of *Rana pipiens*, and the operating stage was the same as that used in previous experiments (Streeter '06, fig. 3, p. 547). This is just at the end of the non-motile stage, and the outer form of the larvae shows a distinct tail bud, and the eminences on the head caused by the optic cup and head ganglia. The ear consists of an invaginated saucer-shaped mass of cells just in the process of being pinched off from the deeper layer of the skin, with the edges inverted and in the process of completing the closure of the vesicle; that is, transition from auditory cup to auditory vesicle. In this, as in previous papers, the attempt is not made to distinguish between these two stages, and the term 'ear vesicle' is used to cover both. The technique of the operation is as follows: Two larvae are removed from their gelatinous capsules and placed side by side in distilled water, under a binocular microscope. With two no. 12 embroidery needles, a linear tranverse incision is made through the ectoderm of one of the specimens over the site of the ear vesicle, the incision being about three times as long as the vesicle, and the right side of the animal always being used for the operation. The lips of the wound are then gently everted, forward and backward respectively, which discloses the thin lateral wall of the vesicle, or if this is torn in the removal of the skin then one sees the pigmented concave interior of the vesicle. With the needles the vesicle is now loosened from its pocket and cast away. This leaves an empty pocket, free for the transplantation. The right vesicle of the second specimen is uncovered in a similar way, loosened from its pocket and then slipped into the empty right auditory pocket of the first specimen. In tucking in the vesicle under the edges of the wound care was taken in all cases to place the vesicle so that the lateral or concave open surface of



the vesicle should lie against the brain, and the median or convex surface toward the opening of the wound. The lateral and median surfaces of the vesicle are easily recognized and therefore this transplantation in a reversed posture can be done with great accuracy. This method of placing the vesicle has a double advantage. It furnishes a complete rotation of 180 degrees and it is a secure way to transplant the vesicle which is very easily wedged in the pocket in that posture. The vesicle shows no tendency to escape, and as the lips of the wound are not very wide apart, it is not necessary to take any precautions about holding them together. They take care of themselves and need no superimposed weights or further attention. The specimen is now set aside and in the course of three or four hours all traces of the wound have disappeared. The specimens are allowed to go on with their development for fourteen days, the period usually necessary for the formation of the canals, at the end of which time they are preserved in a chrome-acetic mixture and are ready for examination. In most of the operations two specimens were utilized, one always being discarded after transplanting its right vesicle. In four experiments the writer succeeded in making a complete exchange of the right vesicle between the two specimens and preserving both of them. This involves great care and the additional effort required does not justify the procedure. The preserved specimens were imbedded in paraffin, cut in series and stained with hematoxylin and congo-red. Examination of them showed that nineteen of the experiments were successful, in that the transplanted vesicle had continued in its development far enough to recognize its different parts and their posture. Wax-plate models after the Born method were made of them all, and drawings of these, together with a photograph of a selected section from each series, are shown in figures 1 to 38. I may here state that these photographs were made with the apparatus belonging to my colleague, Professor Novy, and it gives me pleasure to take advantage of this opportunity to acknowledge his courtesy and his assistance in their preparation.

Other operations were performed with a view to the determination of character and degree of the environmental control, but these

will not be reported on in this paper; we limit ourselves to the question of whether environmental control does or does not exist. It may be pointed out that the above operative procedure offers a complete and severe test for the existence of such control. It is so arranged that we have for the experiment a transplanted foreign ear vesicle, which does not naturally fit in its new pocket and which is intentionally placed in a posture as abnormal as possible.

#### RESULTS OF THE EXPERIMENTS

At the outset it may be stated that in all cases where the labyrinth had developed into a structure with sufficient completeness for the identification of its relations, it was found that the position of its canals and various chambers as regards the surrounding structures was practically normal, in spite of the manipulation it had undergone at the time of the operation. As has previously been shown (Streeter '07, p. 433) the posture of the labyrinth can be determined both by the histological structure of its walls and by its outer form, as determined by wax-plate reproductions. The description of these morphological characteristics will not be repeated here: it will be sufficient to state that they are so definite that the various parts of the labyrinth can be recognized without difficulty, even though they happen to be incomplete or unequally developed.

For the sake of compactness table 1 is annexed in which are detailed the separate features of each of the nineteen transplanted labyrinths as found on examination. Each feature is marked 'normal', 'imperfect,' or 'absent,' indicated by the signs *N*, *I* and *O*. These are used in a liberal sense so, that 'normal' signifies practically normal, and includes structures abnormally large or small; 'imperfect' signifies quite abnormal, the characteristics, however, being sufficiently well defined for identification of the structure; and 'absent' means either completely absent or unrecognizable.

The features as listed will for the most part explain themselves. It may be mentioned, however, that by canal planes is meant the relative position of the canals as regards each other. Planes

drawn through the anterior and posterior canals would meet at an angle of about 95 degrees at the crus commune. This is quite constant in all of the labyrinths. The lateral canal is about perpendicular to the other two. At the end of two weeks the planes are not so well defined as they are later, but when the canals are otherwise normal the planes can be determined, even as early as that.

From a study of this table it will be seen that we can arrange our experiments in three groups, based on the completeness of the

TABLE 1

LABYRINTH No.	POSTERIOR SIZE	ANTERIOR CANAL	POSTERIOR CANAL	LATERAL CANAL	CANAL PLANES	CRUS COMMUNE	VESTIBULE	LAGENA	ENDOLIMPHATIC APPENDAGE	ACOUSTIC NERVE	ACOUSTIC GAN- GLION	CAPITULATING'S CAPSULE
<i>Normal group</i>												
I	N	N	N	N	N	N	N	N	N	N	N	N
II	N	N	N	N	N	N	N	N	N	N	N	N
III	N	N	N	N	N	N	N	N	N	N	N	I
IV	N	N	N	N	N	I	N	N	O	N	N	N
V	N	N	N	N	N	N	N	N	N	N	N	I
VI	N	N	N	N	N	N	N	N	N	N	N	N
VII	N	N	N	N	I	N	N	O	N	N	N	N
VIII	N	N	N	I	N	I	N	N	N	N	N	N
<i>Abnormal group</i>												
IX	N	N	N	N	I	N	N	I	O	N	N	N
X	N	N	I	N	N	N	N	N	O	N	N	N
XI	N	I	N	N	I	N	N	N	O	N	I	N
XII	N	N	N	N	I	N	N	I	O	N	N	N
XIII	N	I	N	I	N	N	N	O	O	N	N	N
XIV	N	I	I	I	N	N	I	I	O	O	N	N
XV	I	N	I	N	I	N	I	N	O	N	N	N
XVI	N	N	I	I	N	N	I	N	N	I	N	N
<i>Doubtful group</i>												
XVII	N	N	N	I	I	N	I	I	O	O	I	N
XVIII	N	N	I	I	N	I	I	I	?	O	N	N
XIX	?	I	O	O	?	?	?	N	O	O	O	N

resultant development of the transplanted labyrinth. These are: firstly, those in which the labyrinth is nearly normal; secondly, those in which the labyrinth is abnormal though the main parts of it are easily recognizable; and thirdly, those in which there might be some question as to the identification of the different parts of the labyrinth. There are eight in the first group, eight in the second group, and three in the last group. Based on their order in these groups we may now proceed to the examination of the individual labyrinths.

*Labyrinth I: (figures 1 and 2).* The photograph shown in figure 1 is taken from Series X 2 C, slide 5, row 3, section 7. The study of this labyrinth shows only one slight departure from the normal, and that concerns the Saccus endolymphaticus which lies somewhat cephalad to the crus commune. It is on this account that it does not show in figure 1, which is directly through the crus. What looks as though it might be the edge of the sac, is the pigment layer of the skin. Aside from its slight anterior displacement it bears the usual relation to the chorioidal roof of the fourth ventricle and is normal in size.

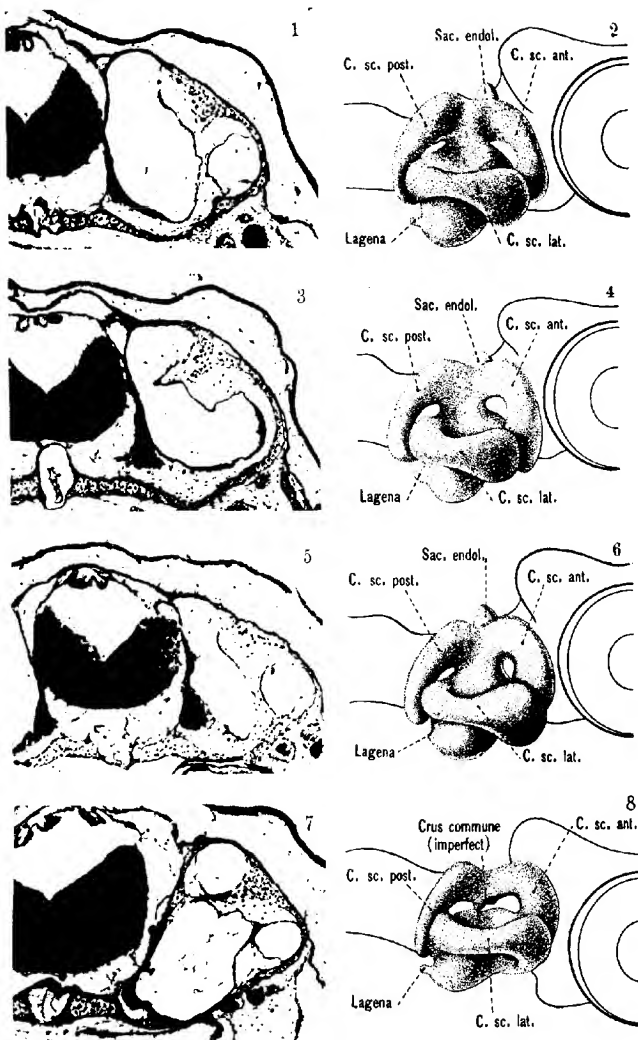
*Labyrinth II: (figures 3 and 4).* Figure 3 is taken from Series X 2 A, slide 4, row 1, section 3. Here as in Labyrinth I the endolymphatic sac is slightly in front of the crus commune. The crus is a little wider than in the former, so both it and the sac can be seen in figure 3. This labyrinth shows no noteworthy departure from the normal in either its histology or general form, aside from the slight displacement of the endolymphatic sac. In the drawing of figure 4 the groove between the ampullae of the anterior and lateral canals has been exaggerated a little more than is warranted by the reconstruction. In figure 3 the thickening of the ventro-lateral labyrinth wall is partly due to the crista of the lateral canal which it represents, and partly is due to the obliquity of the section of the ampulla. The characteristic relation of the endolymphatic sac to the chorioidal roof of the ventricle can be seen. The large ganglion on the ventro-median wall of the labyrinth is the proötic ganglion; the acoustic ganglion lies in a similar position but is found in the more caudal sections. Some of

the acoustic root fibers, however, can be seen entering the side wall of the brain near the endolymphatic duct.

*Labyrinth III: (figures 5 and 6).* Figure 5 is taken from Series X 7 A, slide 4, row 2, section 4. In the reconstruction of this labyrinth there is apparently nothing abnormal. And on examination of the section the only departure from the normal is a slight deficiency in the cartilaginous capsule in the region of the lateral center. The parabasal plate seems normal. The ganglion shown in figure 5 is the proötic near its junction with the acoustic ganglion. The section passes through the endolymphatic sac, the crus commune and the lateral semicircular canal. It shows the intimate relation existing between the endolymphatic sac with the chorioidal membrane.

*Labyrinth IV: (figures 7 and 8).* Figure 7 is taken from Series X 11 B, slide 3, row 1, section 5. This section was selected because it shows the characteristic protrusion of the lagena on the ventromedian wall. The section is toward the caudal end of the labyrinth and passes through, besides the lagena and large vestibule, the posterior canal and the bulging caudal edge of the lateral canal. Sections a little in front of this show the lateral canal opening into the vestibule. Evidently in the process of transplanting this vesicle some injury was inflicted on the cells that were to form the middle part of the median surface. The resultant defect includes the absence of the endolymphatic appendage and a marked imperfection in the crus commune. Otherwise the general form of the labyrinth is quite perfect, as can be seen in figure 8.

*Labyrinth V: (figures 9 and 10).* Figure 9 is taken from Series X 1 B, slide 2, row 3, section 5. The section passes through the endolymphatic sac, the short crus commune, and the lateral canal. The lateral vestibular wall is thin and distended and represents the dropsical type, which is a common deformity. There is a large acoustic ganglion connected centrally with the brain. This section shows well the relation between the endolymphatic sac and the chorioidal membrane. In figure 10 the shading of the lateral vestibular wall makes it look thinner than shown in the model and does not give the swollen appearance it



ought to have. The lateral wall in reality presses directly against the lateral canal. Aside from this dropsical tendency the general form of the labyrinth is quite normal. There is some deficiency in the cartilaginous capsule, particularly in the lateral center.

*Labyrinth VI: (figures 11 and 12).* Figure 11 is taken from Series X 4 A, slide 7, row 1, section 4. This section passes through the endolymphatic sac, crus commune, vestibule with its thickened macula acustica, and the lateral canal. The endolymphatic sac comes in contact with the chorioidal membrane in sections oral to this. Both histologically and in its general form this labyrinth is quite perfect. Figure 11 is the only photograph in the series that was retouched in any way. Here the negative was scratched to show the outlines of the lateral canal and lateral wall of the vestibule more distinctly.

*Labyrinth VII: (figures 13 and 14).* Figure 13 is taken from Series X 7 B, slide 6, row 3, section 3. The section passes through the endolymphatic sac, the crus commune, and the combined vestibule and lateral canal. The lateral canal exists only as a lateral pouch from the general vestibular cavity. A partial separation is indicated by an indentation on its dorsal surface, as can be seen in figure 14. The indentation does not completely perforate the pouch, so the canal is incomplete. There seems to be a general defect of the caudal portion of the labyrinth. So that, in addition to the imperfect lateral canal, the lagena is absent and the posterior canal small though otherwise complete. The acoustic ganglion is large (figure 13) and has a well developed root connecting it with the brain. The endolymphatic sac presents the usual relation to the chorioidal membrane.

*Labyrinth VIII: (figures 15 and 16).* The photograph shown in figure 15 is taken from Series X 2 B, slide 5, row 1, section 2. In the process of embedding and mounting this series, some of the sections were injured, though not enough to interfere with the identification of the different parts. Thus it can be plainly seen that figure 15 passes through the endolymphatic appendage, the crus commune, the vestibular pouch, and the lateral canal. The anterior half of the labyrinth is quite perfect, as can be seen in

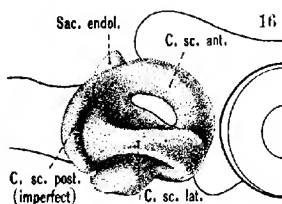
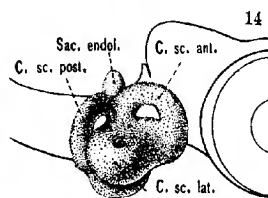
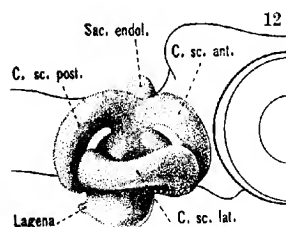
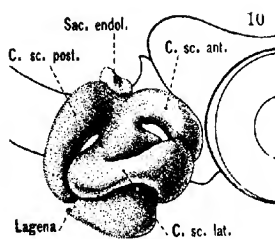


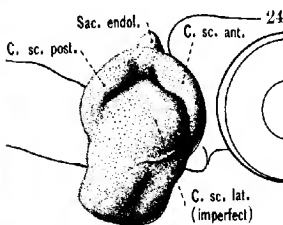
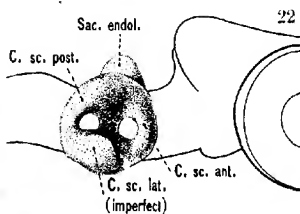
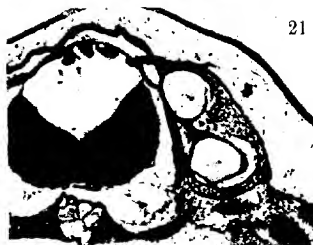
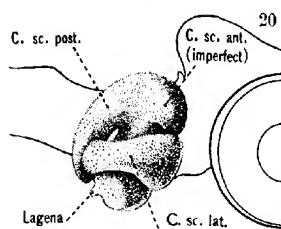
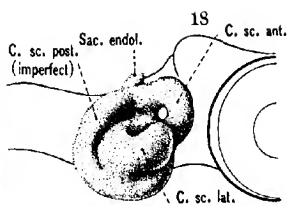


figure 16. The posterior region is defective in that the posterior canal and crus commune consist of a common pouch, taking however the usual shape of the posterior canal. The lagena developed as a pair of short pockets, one extending medially and the other caudally. Apparently the anlage was divided. The endolymphatic sac (figure 15) presents the usual relation to the chorioidal membrane.

*Labyrinth IX: (figures 17 and 18).* The photograph shown in figure 17 is taken from Series X 8 A, slide 2, row 4, section 9. It passes through the endolymphatic sac, the crus commune, and the vestibule with the lateral canal opening into it. In these sections oral to this the lateral canal becomes completely separated, though it is small and is deficient in the region of the ampulla. The anterior canal is small but well formed. The posterior canal is completely formed but lies closely against the vestibular wall. The vestibule is small throughout and lacks the lagena. The acoustic ganglion is well developed and is connected with the brain in the normal way. The endolymphatic sac (fig. 17) bears the usual relation to the chorioidal membrane. This is the first of what we have grouped as abnormal labyrinths.

*Labyrinth X: (figures 19 and 20).* The photograph shown in figure 19 is taken from Series X 11 C, slide 1, row 2, section 8. In this labyrinth the injury involves the endolymphatic appendage and the anterior canal. The latter exists as a blind pouch extending orally from the crus commune. This should be compared with figures 26 and 28, where a similar defect involves the posterior canal. The remainder of the labyrinth is quite perfect. Figure 19 shows the crus commune, the vestibule, with a portion of the acoustic ganglion, and the lateral canal. The endolymphatic appendage is entirely absent.

*Labyrinth XI: (figures 21 and 22).* The photograph shown in figure 21 is taken from Series X 8 B, slide 2, row 2, section 8. Here there is considerable abnormality in the ventral half of the labyrinth, and the labyrinth is correspondingly reduced in size. The anterior and posterior canals and the endolymphatic sac are fairly normal. In figure 21 can be seen the endolymphatic sac with its usual relation to the chorioidal membrane. The



section also passes through the anterior canal just oral to the crus commune and through the vestibular pouch. The latter is much reduced in size. It possesses a macula with a diminutive nerve and ganglion, but the lagena is absent. The lateral canal exists as a lateral pouch projecting from the vestibule (fig. 22). In spite of the abnormal shape due to the deficient vestibule the planes of the anterior and posterior canals intersect at the usual angle.

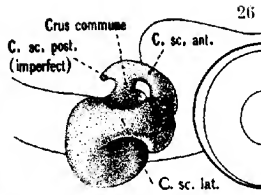
*Labyrinth XII: (figures 23 and 24).* The photograph shown in figure 23 is taken from Series X 7 C, slide 2, row 3, section 8. The section passes through the endolymphatic sac, the crus commune, the vestibule with the large pouch extending laterally from it and representing the lateral canal. The endolymphatic sac bears the usual relation to the chorioidal membrane. The anterior and posterior canals are fairly normal. The vestibule is enlarged and irregular, and illustrates the dropsical type. It possesses the usual macula with nerve and ganglion (fig. 23). The lagena is absent.

*Labyrinth XIII: (figures 25 and 26).* The photograph shown in figure 25 is taken from Series X 4 C, slide 1, row 3, section 5. It passes through the crus commune, the vestibule with its thickened floor, and through the lateral canal just being pinched off from the vestibule. Slightly oral to this the separation becomes complete. On examination of the reconstruction of this labyrinth (fig. 26) it can be seen that the chief injury involves the dorsal part of the labyrinth, and as a result the whole labyrinth is undersized. The greatest defect is in the posterior canal, which is represented by a bud extending caudally from the crus commune. A similar one is seen in figure 28 and the same kind of defect in an anterior canal has already been seen in figure 20. The crus commune and the anterior canal are small though complete. The involvement of the median surface is shown by the absence of the endolymphatic appendage and the lagena.

*Labyrinth XIV: (figures 27 and 28).* The photograph shown in figure 27 is taken from Series X 6, slide 1, row 4, section 2. This labyrinth is of the dropsical or vesicular type. It is possible to recognize its general position but aside from the fairly



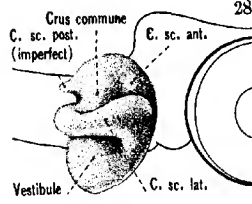
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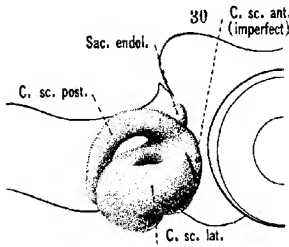
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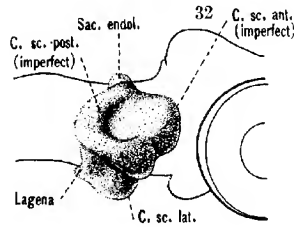
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31



32

normal lateral canal it is poorly differentiated (fig. 28). In this kind of a labyrinth after undergoing differentiation for a short time the process ceases. After that the only further sign of development is an increase in size, which thus produces the dropsical appearance, such as is seen in figure 27. The section is cut obliquely through the oral end of the lateral canal and the anterior canal. This section was chosen because it shows the macula and its nerve ganglion connection with the brain. The anterior canal, like the *crus commune*, is only slightly separated from the general vestibular cavity. The posterior canal is represented by a bud extending caudally from the region of the *crus commune*, such as we have already seen in figures 20 and 26. There is no definite sign of a lagena or endolymphatic appendage.

*Labyrinth XV: (figures 29 and 30).* The photograph shown in figure 29 is taken from Series X 1 A, slide 6, row 3, section 8. It passes through the oral end of the labyrinth, showing the endolymphatic sac, the small anterior canal, and the oral end of the lateral canal opening widely into the vestibule. As can be seen in figure 30, the caudal part of this labyrinth is fairly normal. The oral end is quite imperfect, the parts being small and not properly differentiated. The anterior canal is only a pouch not completely separated from the vestibule. The lateral canal has a deep impression on its dorsal surface marking the beginning of a separation. This labyrinth has not completely recovered its normal posture. It is displaced forwards and is tilted partially forward so that the endolymphatic sac, instead of bearing the usual relation to the chorioid membrane, is opposite the mid-brain and lies embedded in the proötic ganglion. Though considering its abnormal shape it is not far from having the normal posture.

*Labyrinth XVI: (figures 31 and 32).* The photograph shown in figure 31 is taken from Series X 4 B, slide 2, row 2, section 4. The section passes through the endolymphatic sac, which in the sections a little more caudal touches the chorioid membrane in the characteristic way. The section also passes through the *crus commune* and the vestibule with the lateral canal opening into it. The lateral canal is quite perfect, but the anterior and poste-

rior canals are only represented by pouches opening out of the vestibule in common with the crus commune. The shape of the pouches is relatively normal, and they are only deficient in not being pinched off from the vestibule. The lagena is quite normal. The acoustic nerve and ganglion are diminutive.

*Labyrinth XVII: (figures 33 and 34).* The photograph shown in figure 33 is taken from Series X 3 A, slide 2, row 3, section 13. Due to some mechanical or other injury the last three labyrinths in our series are quite imperfect, and from examination of the sections alone (figs. 33, 35 and 37) one would be quite unable to orient them. The reconstructions are of great assistance in this respect. The section taken from Labyrinth XVII was selected because it shows the 'labyrinth fragment' which evidently was a portion of the main vesicle that became detached and developed as a separate little sac (fig. 34, x). In figure 33 it can be seen outside the cartilaginous capsule, partially covered by a cartilaginous coat of its own. It is not connected either by nerve or duct with the main labyrinth. It is possible that in the course of the operation a fragment from the native vesicle was left in the operation-pocket, and eventually formed this structure. Examination of the reconstruction shows that there is an interruption in the lateral canal, its two ends being sealed off. One never finds an open end in these imperfect labyrinths, they are always sealed off. The anterior canal is small but well formed. The posterior canal with the crus commune from a common pouch communicating with the vestibule. The vestibule has a normal macula with diminutive nerve and ganglion. There is no lagena and no endolymphatic appendage.

*Labyrinth XVIII: (figures 35 and 36).* The photograph shown in figure 35 was taken from Series X 3 B, slide 2, row 1, section 11. The whole oral region through which this section passes is of the dropsical type. The caudal region is not so pathological. The posterior canal, though incompletely separated from the vestibule and the crus commune, is otherwise well formed. The caudal half of the lateral canal is fairly normal, but the oral portion is distended like the rest of this portion of the labyrinth. Ventrally in one region this large labyrinth bulges through the parabasal

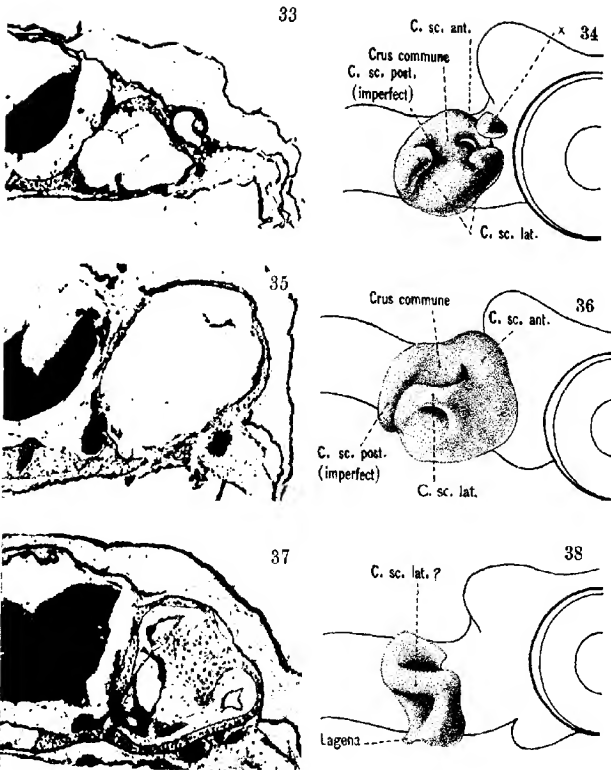


plate and protrudes in the pharynx. On the lateral surface of the anterior canal there is a deep pit indicating the usual site at which it is pinched off from the vestibule and crus commune, otherwise the separation is lacking. The presence of a lagena is questionable, and the endolymphatic appendage is entirely lacking. This, like Labyrinth XVII, through quite abnormal, can be fairly definitely oriented.

*Labyrinth XIX: (figures 37 and 38).* The photograph shown in figure 37 was taken from Series X 11 A, slide 2, row 2, section 2.

This is the last and most imperfect labyrinth in our series. It must be regarded as only a fragment. Apparently there is a lagena with the characteristic finer structure, shape and position. There is also a canal that corresponds in form and position to the lateral canal. Otherwise the different parts cannot be very well differentiated. This labyrinth is included in spite of its fragmentary character because it shows that even such an imperfect labyrinth is apparently affected in its posture by the influences interacting between it and its environments.

In a summary of the first eighteen of these experiments the result that concerns us most is that in all instances where the transplanted labyrinth had developed with sufficient completeness for identification of its various parts it was found that it had almost perfectly regained its normal posture. It was this fact that formed the chief object of our investigation, but we may add the following concerning the resultant abnormalities produced by the manipulation of the vesicle at the time of the operation.

*a.* In 8 out of 18 experiments there developed a practically normal labyrinth;

*b.* Where a defect occurs following the operation it is usually localized in some particular region of the labyrinth; it may be confined to the anterior, posterior, or median portion, while the remainder of the labyrinth is quite perfect;

*c.* The three different canals are defective with about equal frequency (30 per cent), though in an individual labyrinth where the canals are involved the imperfection is not distributed among them equally; and the planes of the canals, whether they are defective or not, are usually normal;

*d.* Defects of the endolymphatic appendage occur with about the same frequency as those of the canals;

*e.* The lagena is imperfect more often than any other part (in 8 out of 18 cases);

*f.* The acoustic nerve and ganglion were always present, though in a few cases quite diminutive; the connection of the nerve with the brain wall can almost always be recognized.



## DISCUSSION

The results that have just been recorded seem to show conclusively that there exists some influence between the transplanted ear vesicle and its environment that tends to control its posture, and that an inverted vesicle is thereby rotated back into the normal position. At the same time we must recognize the fact that Spemann ('10), from investigations of the same problem with experiments of much the same character, came to quite different conclusions. On examining and comparing our results, however, it will be seen that they are not necessarily contradictory, as thought by Spemann, but may perhaps be better described as differing in degree.

Including those in the present paper I have now reported 36 experiments in which the posture of the ear vesicle was especially studied. In 12 of these the ear vesicle had been rotated 180 degrees from its normal position; in 5 of them the ear vesicle had been transplanted without special placement; and in 19 of them the ear vesicle was transplanted and at the same time placed in a definite abnormal posture. In all of these 36 cases the labyrinth regained its normal posture. In Spemann's twelve reported cases in which there had been simple inversion of the ear vesicle, nine of the ear vesicles remained inverted, while two partially and one completely regained their normal posture. The latter Spemann regarded as an unsuccessful operation, surmising that it had slipped back into position directly after the operation. The fact of 'slipping' into the right position is the point of the whole matter. It is the remarkable fact that an ear vesicle, though rotated or turned or transplanted in an extremely abnormal position, nevertheless 'slips' into the correct position, that I am trying to establish in this paper.

It is of interest to examine Spemann's methods in search of some explanation for the difference in our results. For his experiments he used *Rana esculanta* larvae while I used *Rana pipiens*. It is not likely, however, that this would account for the difference in our results. His operations were performed at the time the ear vesicle is in the process of detaching itself from

the deeper layer of the epidermis. This is the same stage used by me. However, in the technique of the operation our methods are different. Spemann raises a relatively large quadrilateral flap of skin, thereby exposing the ear vesicle, which he loosens and inverts so that the anlage of the ductus endolymphaticus points downward. He then replaces the skin flap and secures it in position by a weight, consisting of a slightly curved strip of cover glass. There are two factors here that may account for the difference in our results. In the first place Spemann, by reflecting a large skin flap, exposes a larger area of the deeper structures and perhaps thereby injures the environment in a way that lessens the postural interaction between it and the ear vesicle. It will be remembered in my operations there was only a linear slit opened, which by the spreading of its edges was sufficient for the manipulation of the vesicle. In the second place the use of the weight, as is done by Spemann, to hold the skin flap in apposition may retard the movement of the vesicle and prevent its rotation. From the nature of my wound no weight was necessary.

In addition it may be mentioned that Spemann always placed his vesicles so that the median or convex side remained toward the brain, while in most of my experiments it was made to point lateralward toward the skin. In this respect the displacement of the ear vesicle in my cases was more extreme than in Spemann's, making it all the more difficult for my vesicles to obtain a normal posture. Furthermore, in Spemann's experiments the ear vesicles were replaced in their own original pocket, while in all of my last series they were transplanted to another specimen, here again adding to the difficulty of their postural adjustment. In all other respects there seems to be no essential difference in our methods.

Judging from my own experiments there certainly exists a decided tendency between the ear vesicle and its environment that serves to control the posture, though we know from Spemann's experience that under certain circumstances this tendency is interfered with and the necessary corrective rotation is not accomplished.

The development of a normally placed labyrinth from an inverted vesicle can only occur in one of two ways. Either the ear vesicle at the time of operation consists of indifferent cells which are capable of forming various parts of the labyrinth in accordance with how they chance to lie ('*harmonisch-äquipotentielles System*'); or the ear vesicle itself rotates as a whole after the operation, so that the cells originally intended for the different parts are brought to lie in their correct relation, where they continue in the fulfillment of their destined development. As was clearly argued by Spemann '10, it cannot be explained by the former. All our evidence points to a high degree of differentiation of the cells of the vesicle, and it is conspicuously proven by their possession of laterality, which has been described in the earlier part of this paper. We could not otherwise have a left-sided labyrinth on the right side of the head.

This leaves us with the alternative that the displaced ear vesicle does not stay in the position in which it is placed, but rotates into a normal posture. Regarding the nature of the force that produces the rotation there is yet little information. One must take into consideration at least three possibilities which either separately or in combination may explain its action. In the first place, it may be an active phenomenon on the part of the ear vesicle, that is, intrinsic motility of the vesicle itself; secondly, it may be based upon some attraction existing between some portion of the vesicle and the brain or other structure; and thirdly, there may be some purely mechanical basis for it.

In the first place, regarding the intrinsic motility of the ear vesicle itself, we are familiar with the flowing motion of protoplasm in the case of amoebal pseudopods, and Harrison ('10) has described the remarkable movements of the protoplasmic processes of nerve cells. In these instances there is a movement of one part of a single cell in relation to the rest of the cell. In the case of the ear vesicle, however, we should have to consider a mass-movement of a group of cells. Such movements have already been described for small masses of cells, such, for example, as the lateral line rudiment, which, as has been shown by Harrison ('03) migrates in the course of a few days, all the way from the head,

region to the tip of the tail. Perhaps of a similar character is the shifting of groups of ganglion cells within the central nervous system as described by Kappers ('08) and the author (Streeter '08). These, however, are movements of small clusters of cells.

An example of intrinsic motility of a larger epithelial mass, which will be more analogous to the ear vesicle that we are dealing with, is afforded in the healing of skin wounds of young larvae. The epithelial coat, in such cases, spreads like an elastic sheet from the surrounding area over the wound. This mass-like movement of the epithelial coat is very characteristic. On watching the healing of a denuded area one can see the pigmented epithelial layer gradually spread in from all sides of the wound, covering in, as it does so, the exposed mesodermal elements, until eventually its edges tightly pucker together at the center. This is accomplished not by the formation of new epithelial cells but by the stretching out of the cells already there, as can be plainly seen by the alteration of the pigment pattern. The marked tension on the adjacent epithelium resulting from this movement in the region of the wound is strikingly shown by the way in which the pigment line existing along the dorsal crest of the larva becomes deviated toward the operated side. In such cases the epithelium belonging, we will say, to the left side of the body, is drawn well over to the right side, thus indirectly aiding in the covering of the seat of operation. For an excellent description of the behavior of the epidermis in the healing of wounds in larval *Necturus*, the reader is referred to the paper of Eycleshymer ('07). If the epithelial ear vesicle can move and adjust itself to varying conditions in the same way that the skin epithelium moves and adjusts itself we could then understand the rotation of the vesicles recorded in our experiments.

A second possibility is that the nerve and ganglion mass may serve to draw the vesicle into its proper position. At the time of the operation some of the ganglion element is usually transplanted with the vesicle, and as the latter develops we find the ganglion closely attached to the thickened part of its floor which is to form the macula. At the same time fibers have grown out from the central end of the ganglion to attach themselves to the

side of the brain wall. This tendency to an early nerve-ganglion connection between the ear vesicle and some portion of the brain wall is evident even when the vesicle is transplanted in a strange environment such as in front of the eye (Streeter '06). The nerve seeks the brain wall, and is it conceivable that after it is securely attached it would act as a check or guy rope on the vesicle. With the subsequent growth and the change in the relative positions of the different structures, the nerve attachment would pull on it and, depending on its previous position, would tend to rotate it one way or another. When the vesicle is in front of the eye there are so many abnormal factors present that one could not expect a successful correction of posture through this means. But in the auditory region where other things are favorable it is conceivable that the nerve attachment might at least help in the adjustment of the position of the vesicle. As a modification of this idea it is conceivable that there exists some mutual attraction between the macula, ganglion, and brain wall which tends to draw them together by some psysico-chemical process. This would result in the same effect on the labyrinth as a whole as a tension of the acoustic nerve, and would serve in the same way as an assisting force in the adjustment of the position of the vesicle. In an analogous way it is possible that the endolymphatic appendage may be attracted to the side of the brain wall and thus tend to draw the vesicle into position. Wherever the endolymphatic duct and sac are well formed there exists a constant relation between them and the brain which I have never heretofore seen mentioned. The endolymphatic sac always lies closely applied to the membranous roof of the fourth ventricle near the rhombic lip. Some force may bring these two structures together, and thus we would have a secondary correcting influence on the posture of the whole vesicle. This, it must be admitted, cannot be the whole explanation, as we have vesicles with correct posture in cases where the endolymphatic sac is entirely absent.

As a third possibility there is to be considered, from a purely mechanical standpoint, the shape of the vesicle and the bed or pocket in which it fits. The auditory pocket is bounded on the median side by the relatively firm brain wall, ventrally by the

developing cartilaginous skeleton, laterally and dorsally by the non-resisting auditory capsule and skin. In front of the proötic ganglion mass and the optic vesicle, and caudally the vago-glossopharyngeal complex. These different structures present different degrees of resisting pressure, and thus from different directions there are these compression forces acting upon the ear vesicle, to which, when normally placed, it is properly adjusted by its shape and the firmness of its different parts. Normally there is an equilibrium between the compression forces of the environment and the resisting forces of the vesicle. When the vesicle is abnormally placed there is a disturbance in this equilibrium which continues until the vesicle regains its normal position. Thus we would have the mechanical tendency for the disturbed vesicle to fit itself into the right position. An objection to this explanation immediately suggests itself, and that is the fact that vesicles having an abnormal form, and that could not possibly fit well in the usual pocket, notwithstanding, right themselves almost as well as the normally shaped ones. As another mechanical factor one might think of gravity. It is well known that gravity constitutes a decisive factor in certain embryological processes. Hertwig ('99) and Wetzel ('04) experimentally produced deviation from the normal development of the egg by alterations in gravity through the use of a centrifugal machine. It is true that gravity controls the position within the egg membranes of the amphian larvae in the early stages. The ventral side, due to the yolk mass, is heavier and is always down. In the case of the ear vesicle the large macula in its floor is thicker and presumably heavier than the other portions of the vesicle wall, and we might assume this fact as the reason that we always find the macula towards the ventral side and thereby a factor in the posture of the vesicle as a whole. However, when the larvae are removed from the membranes, as is necessary for purposes of operation, the conditions are quite altered. Being no longer supported by a gelatinous sphere which is easily kept properly erect by gravity, the larvae fall to the bottom of the dish and rest on their side, and we immediately have an abnormal direction of gravity, which persists throughout the critical period in the development of the ear ves-

icle. If gravity were the controlling factor all of the ear vesicles in larvae removed from their membranes would be obliquely placed, whether operated upon or not. This we know does not occur, and therefore we may safely assume that gravity does not exercise any great influence on the posture of the ear vesicle.

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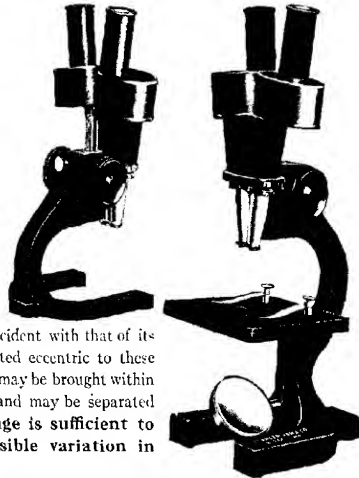
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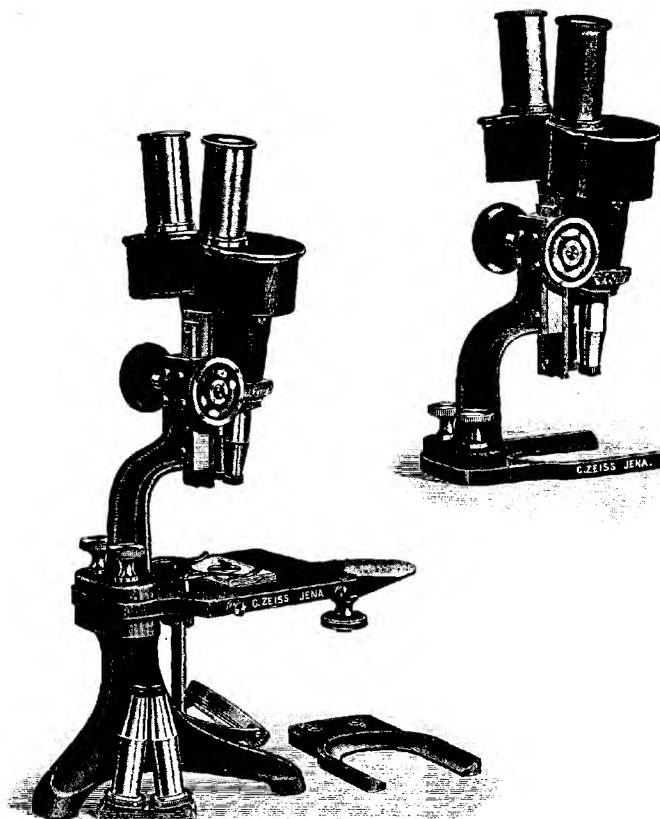
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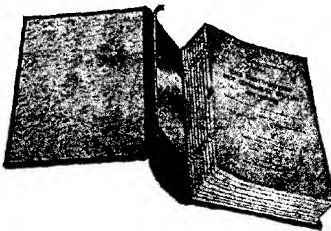
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Since cases of simple Mendelian phenomena have been very frequently described, the interest of the student of genetics has shifted from the attempt to prove, or disprove, Mendelism towards the investigation of the extent of the application of Mendel's fundamental principles. Perhaps this is why the apparent exclusion of anything Mendelian from size inheritance by Castle's work on the ear length of rabbits, aroused so much interest. Castle ('09) found that the ear lengths of rabbit offspring were in general intermediate in relation to the parents. This seemed to indicate a simple blending inheritance in which the size-controlling elements from either parent were permanently joined in the offspring, never to segregate. Yet in the same rabbits Mendelian ratios were being given and segregation was taking place in the color of the hair. The appearance of a paper by H. Nilsson-Ehle ('09) brought a new possible interpretation of the ear length crosses. In this paper evidence was presented to show that there may be two or more Mendelian factors for the same character, which factors develop the character whether they appear alone or in any combination with the others.

Nilsson-Ehle found in certain crosses of oats and wheat in which the colors of the glumes and seeds were considered, that simple Mendelian ratios (3:1) were generally given, but in cer-

<sup>1</sup> This paper is based on an investigation carried on in the Laboratory of Genetics of the Bussey Institution with assistance from the Carnegie Institution of Washington. A full report of the investigation has been submitted to the Carnegie Institution for publication. This statement of some of its main features is published by permission. For the conclusions drawn from the facts recorded the author is alone responsible.



tain strains he obtained consistent ratios which indicated di- and tri-hybrid crosses ( $15:1$ ,  $63:1$ ); in other words, there were two or three factors involved, each of which alone or in combination with the others, produced the same color, and only in the absence of all such factors could the recessive color be seen. The application of this theory to the case of the rabbit ear lengths was immediately made, East ('10), Castle ('11), Lang ('11), by supposing that ear length depended upon several factors each of which behaved as a simple Mendelian unit, but lacked dominance. This would mean that we could see the difference between an ear whose length had been based upon four factors, and one whose length depended upon five or six factors. It would be supposed that the more simplex factors, or doses, there were present, the longer the ear. If the long-eared race bore four duplex factors (8 doses) for length, and the short-eared race bore two similar but not allelomorphic factors (4 doses), the hybrids obtained by crossing these two races would each have six factors in a simplex condition (6 doses). The relative sizes of the two parents and the hybrids would be expressed by the number of doses in each case. The second generation would give rabbits with various numbers of doses and their ears would range all the way from the length of the long-eared race to that of the short-eared race. Unless the two factors in the small race were allelomorphic to two of the four in the large race, there could be found rabbits with ears longer than the long-eared race, and others with ears shorter than the short-eared race, for in the second generation would be formed combinations involving as many as 12 doses and others with none. There would, however, be a much greater number of rabbits with intermediate than with extreme ear-lengths. Just as in a mono-hybrid cross without dominance there are two heterozygous individuals to one of each of the pure types, so in a multo-hybrid cross, where each factor, independent of the others, is giving a  $1:2:1$  ratio, the individuals with all the factors in a simplex or heterozygous condition would be most numerous. The higher and lower grades would appear in decreasing frequency as the extremes were approached (Tammes '11). Now if only a few animals were raised in  $F_2$

it would be expected that they would have intermediate ear lengths. The numbers obtained by Castle were small enough not to show the new combinations expected on this theory.

On account of the many external conditions that may influence the development of the hereditary size factors, one could not expect to find the various grades in  $F_2$  clearly enough separated to discover the number of factors that might be involved. However, evidence favoring a multiple factor interpretation of size would be found in the appearance in  $F_2$  of extremes not found in  $F_1$  or the increased variability of  $F_2$  over  $F_1$ , especially if extremes beyond the parental races appear.

In plants numerous experiments favoring a multiple factor hypothesis have been described. Since definite ratios may be obtained for qualitative differences, such characters afford the most convincing evidence. Supporting Nilsson-Ehle's results, East ('10 and '11), and East and Hayes ('11) in endosperm and pericarp colors of maize, found ratios which are apparently to be explained only by multiple factors. Tammes ('11) crossed two different sorts of blue Linum; an intermediate first generation was followed by a second generation with a wide range of variability, the intermediate shades having the greatest frequencies.

From quantitative differences in color one is led to quantitative differences in shapes and forms. The work of Kajanus ('11 and '12) on the roots of Brassica and Beta affords evidence to support the hypothesis that various form types of these roots are due to combinations of multiple factors for length, or length and roundness. Emerson ('10) reports increase in the variability of the shapes of the second generation from certain crosses with squashes and gourds. These cases of form differences do not show dominance. Shull ('11 b) described a case involving a double gene for the same character, that is dominant. He found that the flat and triangular seed capsule of *Bursa bursa-pastoris* is dominant to the oval and, in section, round seed capsule of its recent mutant *heegeri*. The ratios of the second and third generations strongly indicate that there are two genes involved, each of which can produce the *pastoris* type of capsule.

From quantitative differences in form we come to quantitative differences involving size alone. The possible application to size inheritance of this theory of multiple factors, so strongly indicated by color characters, was soon realized by Nilsson-Ehle ('07). He found that crosses between races of wheat or oats with long and short stalks gave intermediate hybrids whose offspring had stalks ranging all the way from the length of the long-stalked race to that of the short-stalked race. The length of the head of a certain wheat acted in crosses as though it were dependent upon a strong dominant shortening factor and two weaker factors for length, that lacked dominance. Spillman ('02) in crosses involving the length of wheat heads, and Emerson ('11), in certain crosses involving the height of corn plants, report intermediate first generations followed by second generations in which the ranges of variability included, or even exceeded, the extremes of the parental races. East and Hayes ('11) present crosses of maize involving ear length, number of rows per ear, size of grain; Emerson ('10) sizes of beans; Tammes ('11) sizes of *Linum* seeds and petals; in all these cases the same characteristic results were found, namely, a constant and intermediate first generation and a second generation with wide variability. Philips ('12) gives a preliminary report on size crosses in ducks which indicates a like increase in the variability of the second generation birds.

Certain structural characters have been found that seem to depend upon two or more similar units of inheritance. In oats the presence and absence of ligulae, and the arrangement of the spikelets in the head—either on all sides of the rachis or only on one side—give strong evidence of multiple factors, definite ratio being obtained (Nilsson-Ehle '09).

Such physiological characters as winter hardiness, rust resistance and flowering times of cereals have been found by Nilsson-Ehle to show increased variability in crosses. Tammes ('11) found similar results in the opening or remaining closed of ripe *Linum* capsules. They partly open in the first generation; in the second generation some remain closed, others fully open, while most are half opened. Tschermak ('11) and Leake ('11) pre-

sented studies on the blossoming times of peas and cotton. In both plants crosses between late and early blooming varieties gave plants with intermediate blooming time. In the second generation some plants began to bloom as early as the early blooming variety and others as late as the late blooming variety.

Perhaps as strong evidence for the assumption of different factors influencing the same character as is afforded by ratios in the second and third generations, is found in crosses between apparently similar races which give marked variability in the second generation following a first generation that was like the two parents, and no more variable than the parental races. In such cases the characters in the two lines that have been crossed are supposed to be caused by different factors which produce the same effect. In the second generation, where recombinations first have a chance to appear, new grades are found. Cases have been mentioned that show extremes beyond the parental types. In these cases there must have been a different number of factors in each parental race, if we suppose for the moment that the factors are all of equal power. In the following cases each parental race may be supposed to have the same number of factors (although not identical ones) and so the races appear to be alike.

The occurrence of two white races of sweet peas which give colored offspring when crossed is undoubted evidence that two different factors are involved in producing color. In the following cases the factors are supposed to be similar to each other so that their presence cannot be determined until the second generation. Nilsson-Ehle ('11) found two strains of red wheat, which, when crossed together gave whites in the second generation. Whites also appeared when certain lines of black oats were crossed, in the ratio of fifteen blacks to one white. To lines of oats (Nilsson-Ehle '09) whose spikelets were arranged on all sides of the rachis gave, in the second generation, some plants with spikelets on one side of the rachis. Kajanus ('11) reported three crosses between apparently similar races of long beets which gave the same form in the first generation, while in the second generation some very long individuals were obtained. In crossing dif-

ferent strains of cotton of equal height Balls ('07) found a great variability in the heights of the second generation. The first generation was uniformly close to 100 cm., the second generation ranged from 30 cm. to 100 cm. Hayes ('12) has crossed varieties of tobacco with similar numbers of leaves which produced plants with the same number of leaves in the first generation. The leaf number for the plants of the second generation was very variable. Keeble and Pellew ('10) have worked out a very clear explanation of an increase in variability found in a second generation following the crossing of two semi-dwarf races of peas. The parental races were very constantly between 3 and 4 feet tall. The first generation showed increased vigor, but a constant height of 7 to 8 feet. The second generation ranged from  $1\frac{1}{2}$  to 8 feet. This case differs from those preceding in that the factors, to whose recombination the new grades in the second generation were due, did not produce the same effects. One factor increased the distance between the nodes, the other increased the thickness of the stem, and this thickening enabled the internode to attain greater length. One parental race had the factors for thick stems and short internodes, the other had the factors for thin stems and long internodes. The recombinations in the second generation produced plants with long internodes and thick stems (8 feet as well as plants with short internodes and thin stems ( $1\frac{1}{2}$  feet). The combinations found in the parents also occurred. Moreover, the ratios in the four classes closely approximated expectation. In this case height is dependent upon two Mendelizing factors.

In other cases size may depend upon a single factor. Some of these cases are mentioned: tall and dwarf peas, Mendel; tall unbranched habit vs. dwarf branched sweet peas, Bateson and Punnett ('08); axial vs. terminal position of bean pods, Emerson ('04); tall vs. dwarf *Antirrhinum*, Baur ('11); dwarf vs. normal tomatoes, Drinkard ('08); long vs. short styles of *Oenothera*, de Vries ('01, p. 435); long vs. short wings of *Drosophila*, Morgan ('11); long vs. short hair in various mammals, Castle ('05); brachydactylous digits in man, Farabee ('05) and Drinkwater ('08);

short stocky vs. long slender legs of Dexter-Kerry cattle, J. Wilson ('09).

The evidence presented by plant breeders seems to carry conviction. It has shown that the assumption of multiple factors is the most simple theory to explain certain ratios in crosses involving color and its absence. Similar ratios are also found in crosses involving certain structural characters (ligulae and the arrangement of spikelets in wheat). Now if qualitative characters may depend upon multiple factors in certain cases, there is just as good evidence for saying that certain morphological characters depend upon multiple factors. Size crosses differ from crosses involving multiple factors for color and structural characters in that in the latter cases the color or the structure may be absent, and, so definite ratios can be found; whereas in the former size as such cannot be absent, so in most cases no definite ratios can be found. Otherwise, these two types of characters are strikingly similar. Both show wide variations in the second generation not found in the parental or first generations. Both types show similar wide variations in the second generation in crosses between certain strains that appear to be alike and in these cases the first generation is like the parents. Both types may show mono-hybrid ratios in the same sorts of characteristics. Any theory to account for the wide variations in the second generations of crosses involving characters which cannot afford definite ratios must also account for this similarity with cases where definite ratios may be found, and again, with cases where simple Mendelian ratios and complex ones are found for the same character differences. Size and Mendelian inheritance are not incompatible, as is shown by many mono-hybrid ratios from crosses involving size. In one case (semi-dwarf peas) it has been shown even by ratios that size may depend upon two distinct Mendelian factors. Surely the most simple theory that can be given to explain this phenomenon of increased variability, which in itself cannot be doubted, being reported by many investigators for so many characters, is that there exist units of inheritance, introduced by the parents, to the segregation of which units in the germ cells of the hybrids, the new combinations are due.

## EXPERIMENTAL

In 1908 Castle started a second experiment that should test this hypothesis by obtaining larger numbers of animals so that the variabilities of the first and second generation could be compared. As the size of the whole animal was to be considered, crosses were made between a small male rabbit from a small race, the Himalayan, and a series of large females that had been used in the ear length crosses. The pedigrees of the females are known and for several generations there were only slight differences in the weights of the pairs of ancestors. In 1910 the experiment was put into the hands of the writer. By that time the original crosses had been made and weights for growth curves had been recorded for the animals raised up to that time. I wish to acknowledge here a keen appreciation of my indebtedness to Professor Castle for the privilege of completing this work which he had planned and already started, and to express gratitude for the assistance and advice that he has contributed towards the completion of this work.

In most cases the second generation consisted of a back cross of the first generation females to their male parent. A few back crosses of first generation males to their female parent were made and a few crosses between first generation males and females. Measurements were made of the skull and long bones of the rabbits after the bones had attained full size. Fifteen months was set as the age for killing a rabbit, as it was found that the bones were fully grown at that age. Sixteen measurements for each set of bones were recorded.

*a. Coefficients of size*

It was found that there was enough lack of correlation between the various measurements to give different results when different characters were considered, so it became needful to obtain a number for each animal in which the various measurements would be equally represented to express the size of each animal. As the measurements varied from 2 to 10 cm. no absolute average could be used; for a small deviation in a short measurement

would have far greater significance than an equal deviation in a long measurement, and so in any average the large deviations in the long measurements would entirely overbalance the equally important small deviations in the short measurements. The following method was used to obtain a number for each animal, which may be called the coefficient of size (C. S.), a number based on relative deviations. The average of a character for one fraternity was used as a dividend into which were divided the individual measurements of the animals in the same fraternity. The quotients so formed gave a series of ratios expressing the relative sizes of the various sibs above or below their mean. By this method the ratios of the other characters of one animal to the corresponding fraternal means were obtained. The average of these ratios gave the coefficient of size (C. S.) for that animal. These coefficients range from 0.930 for the largest animals to 1.070 for the smallest ones. In classifying them, classes 0.005 in extent were used. From these distributions, standard deviations were calculated. Since the means of all fraternities lie always in the column whose value is 1, it will be realized that the sums of all the individuals in each column will give a correct description of the variability of one generation of a whole family; in other words, different fraternities from the same family may be classified together. The standard deviations of these family distributions will be based on deviations from the various fraternal means. This will give a more accurate result than if the actual deviations had been calculated from the means of all the individuals averaged together, as would be done in applying the formula for standard deviation to the values themselves. It must be supposed that the means of all the fraternities do not coincide. The mean of all the values would give the extreme variates even greater deviations and the standard deviations would be higher than those obtained by this method. It will be seen now that the coefficients of size of all the animals in one generation may also be classified together.

In the tabulation of these coefficients of size it is at once observed that all the most extreme coefficients belong to individuals



from the back cross. Naturally both the  $F_1$  and the back cross offspring occur in greatest numbers about the middle class and decrease in either direction away from this class. But the back cross extends much further than the first generation. It is unfortunate that the numbers of the  $F_1$  are very much smaller than those of the back cross. It may be claimed that with larger numbers more extremes would have been found in the  $F_1$ . This is undoubtedly true, but it is highly improbable that any individuals that approached the back cross extremes would have

TABLE 1

*Standard deviations of coefficient of size of the first ( $F_1$ ) and back cross (B.C.) generations in the various families. The numbers of individuals are given for each fraternity (No.).*

FAMILY	$F_1$	NO.	B.C.	NO.
647	0.0145	4	$0.028 \pm 0.002$	25
1443			$0.026 \pm 0.003$	17
1471	0.015	7		
1491	0.015	4	$0.019 \pm 0.001$	24
1493	0.009	13	$0.021 \pm 0.001$	60
1531			$0.027 \pm 0.001$	45
1532			$0.027 \pm 0.003$	15
1537			$0.020 \pm 0.001$	46
2011	0.019	5	$0.025 \pm 0.003$	11
1493 $\times$ 2379	0.019	14	$0.017 \pm 0.001$	31
Total.....	$0.014 \pm 0.001$	33	$0.023 \pm 0.0007$	243

This family is not from a size cross. 1s is used as a check, so is not included in the totals.

been found. The standard deviations show that there is a real difference in the variability of the two generations. In table 1 the standard deviations in terms of the coefficients of size are given for the two generations, one family at a time as well as for the totals of each generation. In every family resulting from a size cross the standard deviation of the back cross exceeds that of the first generation. Family 1493  $\times$  2379 was carried along as a control as the parents were nearly the same size. It will be noted that the standard deviation of the back cross, in this case,

is slightly smaller than that of the first generation. In families 1443, 1531, 1532 and 1537 there were not enough  $F_1$  individuals to use in determining coefficients of size. The back crosses of these families are given for comparison with the first generations and back crosses of other families. In family 1491 the standard deviation of the back cross is the same as the highest first generation standard deviation, namely, that in family 2011. In 1493 and 1537 the back cross standard deviations are very nearly as low as the highest first generation standard deviations, but the standard deviation of the first generation of family 1493 is much lower than that of the back cross of the same family. This is the largest family obtained.

*b. Classification in relation to the parents*

The data were next treated in a way to show graphically the size relations between the parents, the first, and back cross generations, considering one character at a time. This method of treatment also shows the relative variability of the first and back cross generations and offers another method for combining different families. This method may be called classification in relation to the parents. For each family 15 classes were formed between the parental classes. Each parental measurement is taken as the middle of a parental class; the extent, or range of each class equals one-sixteenth the difference between the parental measurements. Into classes so formed the first and back cross generations were separated. This was done for all characters in all families. From these classifications the number of animals of all families that fall half way between their parents in regard to any measurement is readily found by adding the frequencies in the middle column; likewise the sums in other columns show the distribution of animals in other relations to their parents. The male parent in all families was the same animal. The female parents differed in size to some extent. This means that in the various families and in the totals, individuals are put together that are not exactly similar as to size. They are, however, similar as to their relative positions between their respective parents.

In general the size of all the measurements of the first generation is plainly intermediate, although the modes and means of the various frequencies are markedly above the midparental. This high distribution may readily be interpreted as the effect of increased vigor from the cross. Similar plant crosses show a like increase in size in the first generation (see East '09, Shull '09, Darwin '76). A marked difference in variability is seen in these first generation frequencies when different characters are compared. When the range is wide it is as much extended in one direction as the other, showing that the extreme lows are not to be accounted for by underdevelopment, as an asymmetrical variation might suggest. Such a difference is found when the distribution for the ulna, which has a wide range, is compared with the distribution of the skull length, which has a very limited range (table 2). Four of the remaining measurements are similar to the skull length; two are intermediate; six are wide, slightly less than the ulna. Yet these frequencies include the same individuals. Pearson ('02) found similar inequalities in different parts of the human body. Hatai ('07) found greater variability in the length of the nasal bone and in the zygomatic width in skulls of albino rats than in any other skull characters.

The variations in the ranges of distributions of the different characters, described for the first generation, are also found in the back cross. The means of the back cross fall near the class half way between the midparental (middle class) and the small parent to which the first generation females were back crossed. A very marked feature of these back cross frequencies is the number of measurements that are as low and lower than the small parent, and as high as, and higher than the mode of the first generation frequencies in which their mothers are included. Since the father and grandfather in all the families was the same rabbit, the actual values of the male (small) parental class in all families is the same, and the values of the adjacent classes differ very slightly in the different families. This means that all animals falling in classes near his class have very nearly the same sizes in the different families. This makes the occurrence of these short measurements the more convincing evidence of segregation.

TABLE 2  
Classification in relation to parents based on skull lengths and "na lengths, to show the relative variability of the two measurements and of the first generation (F<sub>1</sub>) and the back cross (B, C.)

[illegible]

with assistance from Mr. S. Wright standard deviations were calculated for the total first generation and back cross frequencies, as classified in relation to the parents, for each measurement (table 3). These constants do not give actual deviations because the totals are classified in a purely relative manner. However for purposes of comparing the two generations these standard deviations are permissible because the frequencies in both generations are classified in relation to the same parental classes.

TABLE 3

*Means and standard deviations of the first generation (F<sub>1</sub>) and back cross (B.C.) based on classifications in relation to the parents, similar to those given in table 2 and expressed in terms of those classes.*

MEASUREMENT	MEAN F <sub>1</sub>	MEAN B.C. ♂	σ F <sub>1</sub>	σ B.C. ♂
skull length, total....	10.80	5.55	1.94 ± 0.15	2.60 ± 0.08
skull length, partial, 1.	10.56	5.24	2.76 ± 0.21	2.69 ± 0.08
skull length, partial, 2.	10.02	5.28	2.50 ± 0.19	2.80 ± 0.09
skull width, anterior...	8.39	2.01	3.17 ± 0.24	3.52 ± 0.11
skull width, posterior...	11.00	2.17	3.63 ± 0.27	4.58 ± 0.14
teeth.....	10.12	5.69	2.56 ± 0.19	2.88 ± 0.09
nasal.....	9.20	4.35	3.47 ± 0.26	2.87 ± 0.09
mandible 1.....	10.27	4.75	2.04 ± 0.15	2.99 ± 0.09
mandible 2.....	8.95	3.16	2.90 ± 0.22	3.29 ± 0.10
humerus.....	12.20	5.86	3.65 ± 0.27	3.55 ± 0.11
ulna.....	12.24	5.27	5.43 ± 0.42	4.61 ± 0.15
femur.....	11.83	5.54	3.85 ± 0.30	3.38 ± 0.10
tibia.....	11.90	6.16	4.07 ± 0.31	3.98 ± 0.12

Six measurements show significant increase in the back cross over the first generation, three show decrease in the back cross, and four lie within the probable error, meaning practically no change. This is a mathematical demonstration that there is enough lack of correlation between the bone measurements for different characters to give different results. Since twice as many characters show increase as decrease, it can be concluded that there is greater variability in the back cross offspring than in the offspring of the first generation.

*c. Body weights*

When the work was taken over by the writer it was believed that body weight could be taken as a measure of size. Accordingly weights of the rabbits were recorded weekly. From these records growth curves were plotted by which the adult weight for each animal was to be determined. That this could not be done with any nicety, was clearly demonstrated by the study of some 300 curves. Rabbits' weights are very sensitive to changes in conditions; and to obtain curves that would be smooth enough to determine adult weights with accuracy would require more perfect experimental conditions than it has been possible to obtain in raising large numbers of animals. In many cases fat is deposited in such a way that there is no flattening of the curves at about 150 days, as in most cases; instead it may continue to rise for a year without flattening. Pregnancies and nursing disturb the curves of the  $F_1$  females.

The most important information the growth curves afford is their vouching for the recovery of animals that have been sick. In spite of fluctuations, one can see in nearly every one the trace of a regular curve. Through a recognition of the normal type of curve it soon became possible to determine whether at a certain point in a curve an animal was above or below its normal. Based on this element of regularity, which, when shown by a part of a litter, gives the type of curve the other would be expected to have followed, adult weights were estimated. Approximations were made within 100 grams; in a few cases within 50 grams. Coefficients of variability were calculated for the  $F_1$  and back cross fraternities (table 4). Since the means were absolute, standard deviations could not be used in making comparisons. In ten fraternities the C. V. of the back cross animals are higher than any of those of the first generation fraternities. In five fraternities the coefficients of variability are lower than the highest coefficients of the first generation. The weights show, then, that greater variability is found in many of the back cross fraternities than in any first generation fraternity. Whereas by themselves

TABLE 4

*Coefficients of variability of weights, arranged by generations. Each fraternity is considered separately in the back cross (B.C.)*

FAMILY	C. V. of F <sub>1</sub>	NO.	C. V. of B. C.	NO.
647	5.16	4	10.52	5
			5.58	6
			10.27	7
1471	4.09	7		
1491	6.22	4	10.46	10
			6.32	7
1493	8.75	15	13.56	8
			8.30	8
			9.29	12
			9.94	6
1531			13.46	16
			10.45	14
1532			10.27	10
1537			7.35	10
			8.23	13
2037			10.83	23

these weights might bear little or no conviction, on account of the roughness of their estimation, as cumulative evidence in connection with the results based on bone measurements, they certainly may be considered.

#### CONCLUSION

The conclusion to be drawn from these observations seems clear. The back cross is more variable than the first filial generation. This appears in the relative distributions of the coefficients of size of the two generations, whether compared by observation or by standard deviations: it is found when the two generations are classified one character at a time, in relation to the original parents, whether single families or whole generations are considered: and finally the coefficients of variability of the estimated body weights support the same conclusion. There occur characters among the back cross offspring that are smaller than the corresponding characters in the small parent and others that are larger than the modes of the first generation large parents.\*

This conclusion is very similar to many of the cases cited above. All of these deal with heritable characters quantitatively different. They are subject, to a greater or less degree, to fluctuations that, not being heritable, may be roughly assigned to environment. Offspring from crosses between extremes are generally of an intermediate nature. In the following generation new forms appear that are similar to the original parents or even more extreme. The greater number of individuals are intermediate. In certain cases crosses between similar lines, after a first generation like the parents, give a second generation in which a wide range of grades appear. These are the facts that can be definitely ascertained from the work that has been done. On the probability that these same phenomena will always be found, a law may be stated: the second generation of a size cross will show greater diversity than does the first generation or the parental lines. All practical application will come from this.

The interpretation of multiple factors can be applied to all the facts. It goes hand in hand with the mutation, and pure line doctrines of de Vries and Johannsen, and in its breadth of application, and its comprehensive simplicity, this theory, based on the assumption of the segregation of distinct units, is very attractive; by its use as a working hypothesis important facts have been discovered; its acceptance and further development will help to establish a broad and unified system of heredity.

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## THE REACTIONS OF NORMAL AND EYELESS AMPHIBIAN LARVAE TO LIGHT

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### TWO FIGURES

A great deal has been written about the photic reactions of amphibians, but this has been concerned chiefly with the adults, the larval forms having received but scant notice. In the spring of 1913 a series of experiments were carried out upon the larvae of *Rana pipiens*, *R. sylvatica*, and *Amblystoma punctatum* to determine whether they were sensitive to light, and if so, whether they were sensitive to light received through the skin as well as to light received through the eyes.

I take this opportunity to express to Dr. R. C. Harrison my thanks for suggesting this piece of work to me, as well as for his continued interest and criticism.

Banta and McAtee ('06, p. 71) have noted that the larvae of the cave salamander are much more responsive to light than are the adults, and that the younger larvae are more responsive than the older. Both larvae and adults are negatively phototactic. Eycleshymer ('08) found that *Necturus* larvae were negatively phototactic, both in their natural environment and in the aquarium; furthermore, that they orient in a definite way, such that the light falls with equal intensity upon the two sides of the body.

The only other mention made of the reactions to light of amphibian larvae is that by Franz ('10 and '13). Franz describes tadpoles as being indifferent to light—non-phototactic—except when they are crowded into a small space, under which conditions they will all orient themselves to the rays of light, so that their heads are directed toward the source of light. Franz cites this case as one of the numerous examples of so-called phototaxis,

which he has called a 'Fluchtbewegung,' caused by the abnormal conditions under which the animals find themselves.

My experiments were carried on in a basement dark-room, the temperature of which varied between 15° and 19°C. A 60-watt Mazda lamp on a 110-volt circuit was used as the source of light. This was placed in a wooden box, in one end of which there was an opening 8 cm. square, through which the light was projected. The larvae whose reactions were to be tested were placed singly in a flat cylindrical glass dish, of 22 cm. diameter, and 8 cm. deep, holding 2500 cc. of water. The sides and bottom of the dish were covered with black paper, except on the side toward the light where a small window, 3 cm. wide, and extending from near the top to the bottom of the dish was cut. The dish was placed so that its middle point was at a distance of 50 cm. from the lamp, at which point the light had an intensity of about 192 candle-meters. Between the lamp and the dish, and 15 cm. from the former, a screen with an opening 3 cm. high, and 1.5 cm. wide, through which the light passed, was set up.

The larvae used varied somewhat in size, but at the beginning of the experiments the tadpoles were about 12 mm., the *Amblystoma* larvae about 18 mm. long, none of them being shorter than 15 mm. During the course of the experiments, which were begun early in April, and continued through May and part of June, the larvae grew in size, until the tadpoles were about 20 mm., the *Amblystoma* 30 mm. long.

In order to find out whether the larvae were sensitive to light received through the skin, it was necessary to remove their eyes. The method used for removing the optic vesicles was that described by Lewis ('04, '05 and '07) and by LeCron ('07). The instruments used were a pair of very finely ground Noyes iridectomy scissors, and a fine pointed pair of forceps for holding the embryos. The stage at which the tadpoles were operated is that figured by Harrison ('04, p. 201) when the tail bud is just beginning to be perceptible (fig. 1). The *Amblystoma* embryos were operated at the corresponding stage (fig. 2). All the embryos were operated under a binocular microscope. They were placed in watch glasses in a 0.2 per cent normal salt solution, in

which they remained until the wounds had healed, after which tap-water was gradually added. When the larvae had attained a certain size they were transferred to battery jars, each larva being placed in a single jar, which was numbered.

All the larvae tested were kept separate in small battery jars about half full of water and in which a few water plants of various kinds were placed. The frog tadpoles will feed upon the

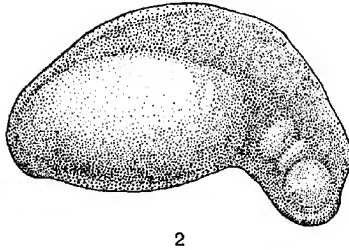
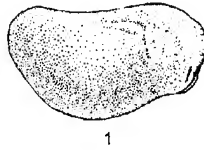


Fig. 1. Embryo of *R. sylvatica*, to show the stage of development used in the beginning of the experiments (after Harrison).  $\times 9\frac{1}{2}$ .

Fig. 2. Embryo of *Amblystoma punctatum*, to show the stage of development used in the beginning of the experiments.  $\times 9\frac{1}{2}$ .

leaves of these plants but the *Amblystoma* larvae will not. The *Amblystoma* larvae were fed regularly during the course of the experiments on small crustaceans which they devoured eagerly, the blinded larvae seeming to have very little difficulty in seizing their quickly-moving prey. The jars were numbered, so that the reactions of the individual larvae could be followed. It was found necessary to isolate the *Amblystoma* larvae early for

the reason that when several are kept together in a single dish they will nip each other's gills, legs and tails, rendering themselves unfit for experimentation.

The larvae were tested in groups of ten, and each individual in a set of tests was given in order a single trial, until each one of the ten had been tested once. This was then repeated until each had had ten trials. A single set of tests, therefore, consisted of 100 trials. The larvae were always dark-adapted, being placed in the dark-room during the afternoon of the day before the experiments were made, and therefore subjected to a condition of darkness for at least fifteen hours before being tested.

Some little difficulty was experienced at first in placing and orienting the larvae in the dish in which they were tested. But after a little practice, it became comparatively easy by means of a large pipette to place a larva in the middle of the dish and at right angles to the impinging rays of light. During the ten reactions that each individual was given in a set of tests, care was taken that both sides of the larvae should be exposed to the light, but no attempt was made to expose the opposite sides in consecutive trials. A larva after having been oriented properly was allowed five minutes in which to react. If at the end of that time it showed no response, it was replaced by another.

It was soon found that the frog tadpoles, both normal and blinded, do not respond to stimulation by light. They were tested both in daylight and in artificial light, and in neither do they show any reaction. As Franz holds ('10 and '13) frog tadpoles are non-phototactic. Franz's experiments of placing a few tadpoles in a small dish and floating this on the water in a larger dish containing tadpoles which show no orientation to light was repeated. But in no case did these tadpoles show any orientation to the light, but continued actively swimming about in all directions, colliding with the sides of the dish and with one another. A single individual placed in the small dish behaved in the same way.

The *Amblystoma* larvae behave very differently. They are sensitive to light and respond to its stimulus by very definite movements. As has been mentioned, the larvae were given five

minutes in which to react, but the reactions were usually quick and definite, the reaction time varying between ten and one hundred and twenty seconds, the average being about fifty seconds. Sometimes a response did not take place for a longer time, but in the majority of such cases it was difficult to say whether the movement was made with reference to the light. Such reactions were therefore called indifferent, that is locomotion straight ahead, or nearly straight ahead, without apparent reference to the light. The typical positive or negative reaction consists, in the first place, of a turning of the anterior end of the body toward, or away from, the light. The turning is usually assisted by the fore-legs, though sometimes they are relaxed and are dragged passively, in which case the turning must be brought about solely through the contraction of the body muscles. This turning of the anterior end of the body is usually followed by a swimming, or creeping on all-fours, in the given direction. Sometimes, however, a larva remains bent in a curve for several seconds, with its anterior end in the given direction and with its posterior end still at right angles to the rays of light. In a few cases this constituted the entire response, the larva remaining in this position for five minutes. In a few other cases, always positive, the whole response is carried out so rapidly that no distinction between turning and swimming toward, or away from, the light could be made. There were, in addition, cases where a larva after having been oriented remained absolutely still for five minutes. In this event it was replaced by the next in order.

In the reactions of the same and different individuals there were the usual inconstancies which one observes in the study of the light reactions of almost any animal, and which have been referred to, for want of a definite explanation, as being due to the changing physiological conditions of the organism. In some cases an individual in a set of ten, on one day would be persistently negative, indifferent, or show no reaction, while two or three days later it would be as persistently positive. Again an individual might show no reaction within five minutes for one trial, and thirty minutes later it might be decidedly positive.

Although the results of the reactions of the individual larvae were kept separate, they were throughout so similar that there is no need to give them separately and they are given combined, in table 1. Five groups of ten larvae each were tested for the reactions of normal individuals, and four groups of ten each for those of the eyeless. It is seen, by referring to the table that, in the case of the normal larvae, out of a thousand reactions, 786 were positive, and 127 negative, while 54 were indifferent, and 33 gave no reactions within five minutes. In percentages these results give 79 per cent positive, 13 per cent negative, and only 5 per cent indifferent, and 3 per cent no reactions within five minutes.

TABLE 1  
*Photic reactions of normal and eyeless Amblystoma larvae*

REACTIONS	CONDITION OF LARVAE							
	Normal				Eyeless			
Directions of.....	+	-	±	0	+	-	±	0
Number of responses.....	786	127	54	33	706	150	79	65
Per cent of responses.....	79	13	5	3	71	15	8	6

The eyeless individuals are almost as decidedly positively phototactic as the normal are. Here there are 71 per cent positive and 15 per cent negative, a decrease of 8 per cent, compared with the normal larvae, in positive responses and an increase of 2 per cent in the negative. There is, however, a larger percentage of indifferent reactions and more of them do not react in five minutes, than in the case of the normal larvae.

These results show that the larvae of *Amblystoma punctatum* are positively phototactic in response to stimulation received through the skin as well as to stimulation received through the eyes and the skin. These results are in accord with what has been found by many investigators of the light reactions of the amphibians, that is, that the eyes are not essential for the light reactions, for the latter may be brought about by stimulation through the skin. Parker ('03), Pearse ('10), and Laurens ('11) have reviewed the literature of this subject, and there is

no need to do so again here. It has also been shown that the phototactic reactions of amphibia when the skin alone is illuminated are apparently not due to the direct stimulation of the central nervous system by light. Parker ('03) has shown this in the case of the frog, while Dubois ('90) on *Proteus*, and Reese ('06) on *Cryptobranchus* and *Necturus* have done the same for these animals. *Amblystoma* larvae are, of course, much smaller and more transparent than the adult forms which these investigators used, and the chances that the light, penetrating the muscles, might directly stimulate the central nervous system are much greater. But further series of experiments bring additional evidence that here again the reactions are not due to the direct stimulation of the central nervous system. In these experiments the testing dish was placed at a distance of 25 cm. from the light,

TABLE 2  
*Reactions of normal Amblystoma larvae to local skin illumination •*

REACTIONS	REGIONS ILLUMINATED											
	Head				Mid-body				Tail			
Directions of.....	+	-	±	0	+	-	±	0	+	-	±	0
Number of responses.....	123	31	22	24	107	26	36	31	110	32	31	27
Per cent of responses.....	62	15	11	12	54	13	18	15	55	16	16	13

where its intensity was about 768 candle-meters. The beam of light was made to pass through a small opening in a screen placed close to the glass dish. In this way a small area of a larva could be illuminated. A series of tests were made on ten normal and on ten blinded larvae, in which different small areas were illuminated. Three regions were selected which may roughly be described as the head region, the ventro-lateral mid-body region, and the tail region. Fifteen minutes was the time allowed for the responses. The results of these tests are given in tables 2 and 3. As may be seen, positive responses were obtained when each of the three regions in both normal and eyeless larvae were exposed to the light. A simple turning of the anterior end of the body toward or away from the light was taken as a positive or negative response. The beam of light was, of course, so small,



TABLE 3

*Reactions of eyeless Amblystoma to local skin illumination*

REACTIONS	REGIONS ILLUMINATED											
	Head				Mid-body				Tail			
Directions of.....	+	-	=	0	+	-	=	0	+	-	=	0
Number of responses.....	112	35	27	26	106	30	35	29	108	38	25	29
Per cent of responses.....	56	18	13	13	53	15	18	14	54	19	12	15

that a movement in its path toward or away from its source was impossible. The percentages of indifferent reactions and of no reactions within fifteen minutes were high as compared with the results obtained when the entire eyeless animal was illuminated. The head region in the normal larvae proved to be more sensitive than the others, due to the fact that the retina was also illuminated. In the blinded larvae there was no evidence that one region was more sensitive than the others, which is not in agreement with the results of other investigators on other forms. Dubois ('90) found the tail of *Proteus* to be the most sensitive region of the skin, and both Reese ('03) and Pearse ('10) found the same for *Cryptobranchus*. Reese also noted that the head of *Necturus* was more sensitive than the tail, due probably to stimulation received through the eyes, and Pearse showed that when the eyes of *Necturus* were removed the tail was the most sensitive region. Eycleshymer ('08) also found that in both normal and decapitated *Necturus* larvae the tail is the most sensitive region of the skin.

As has been seen the larvae of *Amblystoma punctatum* are positively phototactic, both in the normal and eyeless condition. The adults, on the other hand, are negative, both when normal and when blinded (Pearse '10, p. 173). We have here then an example of an animal changing the sense of its reaction to light from positive to negative with its change from the larval stage to the adult.

The skin chromatophores of amphibian larvae have been shown to present different conditions of expansion and contraction of their pigment under different conditions of light and darkness.

Babak ('10 and '13) has recently pointed out that normal and eyeless *Amblystoma* larvae show differences in pigmentation according to the illumination and background. The dark condition of the larvae, in which the pigment cells are expanded, can be produced in three ways, namely, (1) by placing normal larvae in diffuse light on a black background, (2) by placing normal larvae in darkness, and (3) by placing blinded larvae in diffuse light. The light, or pale condition of the larvae in which the pigments cells are contracted, can be produced (1) by placing normal larvae in diffuse light, on a white background, (2) by placing blinded larvae in darkness. It occurred to me that perhaps the condition of the pigment might affect the sensitiveness of the larvae to light, in that in an individual in which the pigment was contracted, the nerve terminations in the skin might be more easily stimulated than in one in which the pigment was expanded. A series of comparative tests were, therefore, carried out with the object of finding out whether there was any difference in the sensitiveness of dark and pale animals, and also whether adapting the larvae to darkness or to light affected their photic reactions. Two groups of normal and two groups of blinded larvae consisting of ten each were selected, and were exposed in turn to the several conditions of illumination and background mentioned above. Battery jars entirely covered with white paper were used to obtain a white background. Babak's results were verified. Flemming ('97) obtained somewhat similar results with normal salamander larvae. He found that if they are placed in darkness they become dark, and that if they are placed in bright light in white porcelain dishes, they become pale and transparent. Fischel ('96) obtained exactly opposite results with the larvae of *Salamandra maculata*, which he found to become pale when kept in darkness, and dark when kept in the light. Fischel's results agree with those of Hermann ('86) on frog larvae, a condition exactly opposite to what is found to take place in the adults.

In my experiments the dark-adapted larvae were kept in the dark five to seven days before they were tested. The larvae which were light-adapted were allowed to remain three to five days in bright diffuse daylight, and were tested only in the after-



to light. But the reactions to light are very different according as the larvae have been adapted to light or darkness. The normal larvae which have been kept in the dark, and are, therefore, darkly pigmented are very much more sensitive than are those which have been allowed to remain in the light. When darkly pigmented larvae, which have been kept in the dark are compared with darkly pigmented larvae which have been kept in the light on a black background, it is seen that the latter are much less sensitive to light, and show a smaller percentage of positive responses, and a much larger percentage of indifferent reactions and of no reactions. The pale larvae which have been kept in the light on a white background are also much less sensitive to light than the darkly pigmented ones which have been kept in the dark, and are equally lacking in sensitiveness to those darkly pigmented larvae which have been kept in the light on a black background.

The eyeless larvae show also that those which have been kept in the dark are more sensitive to light, giving a larger percentage of positive responses than those which have been kept in the light.

In both cases, then, it may be said that dark adaptation brings about a condition of greater sensitiveness to light, irrespective of the condition of the pigmentation in the skin. The retinal pigment reacts to light probably in the way that it usually does, that is to say, when the animal is kept in the dark, the pigment migrates proximally, and when the animal is kept in the light, it migrates distally. In the dark-adapted animal then it might be assumed that, since the pigment was contracted, the retinal elements were more sensitive to light, and that the reverse might be said of the animal kept in the light. The condition of the retinal pigment probably does affect the sensitiveness of the normal larvae, although, as we have seen, the skin pigment does not have any influence on the sensitiveness of either the normal or the blinded larvae. It has been found that in some amphibians there is a connection between the nerve terminations of the eyes and those of the skin, as is to be inferred from results when the skin is illuminated. Engelmann ('85) found that changes

took place in the retinae of frogs when only the skin was exposed to light; though Fick ('90) obtained results which led him to conclude that interference with the normal respiration was the cause of these changes. Koranyi ('92) noted that illumination of the skin caused microscopic changes in the retina similar to those produced by illumination of the eye itself. In my results there is also an indication that there is such a connection between the nerve terminations of the eyes and of the skin. As has been pointed out there is a difference in the condition of the skin pigment of normal and blinded larvae under the same conditions of illumination and background. If normal larvae are placed in diffuse light on a white background they become pale (that is, the pigment in the skin chromatophores contracts); if blinded larvae are placed under the same conditions, they become dark (that is, the pigment expands). If normal larvae are placed in darkness they become dark; if blinded larvae are placed in darkness they become pale. If normal larvae are placed in diffuse light on a black background, they become dark, if blinded larvae are so placed they also become dark. In only this last case then of placing normal and blinded larvae in diffuse light on a black background are the same conditions of the skin pigment obtained. As we have seen however, in the blinded larvae, the nature of the background is of no importance, since both white and black backgrounds in diffuse light produce the same results, light and darkness being the only factors to be considered in the case of the blinded individuals. Normal larvae, on the other hand, show different conditions of pigment when placed in diffuse light on a white and on a black background. It would seem fair to conclude then that there must be some connection between the nerve terminations of the two light receptors, the eye and the skin, since in the larvae in which the eyes are left intact a different condition of the pigment of the skin is obtained from that which is found in the blinded larvae under the same conditions of illumination and background.

For the experiments which have been so far described none of the *Amblystoma* larvae were under 15 mm. in length. They all showed a sensitiveness to light, giving positive responses. At

this stage, then, all the larvae have already acquired the sensitivity to light. It seemed worth while to attempt to find out at about what stage in the development of the larvae this sensitivity first made its appearance. Accordingly ten young normal and ten young blinded larvae were selected, and their responses to light tested. They were placed at a distance of 25 cm. from the lamp where the light had an intensity of about 768 candle-meters. A series of readings of a thermometer placed in the water in the testing dish showed that the increase in the temperature of the water after the lamp had been burning for a period of six hours, was less than a quarter of a degree centigrade. The temperature of the room, it may be mentioned, usually rose about 1.5°C. during such a period.

These larvae when first selected were between 7 and 8 mm. long, responding to mechanical stimulation by rapid swimming movements. At this stage they were, however, insensitive to light, remaining absolutely motionless, at right angles to the rays of light. It was not until the larvae had reached a length of about 11 to 12 mm. that they showed any response.

At this stage the gills are well-developed and tufted, the balancers are fully developed, and the fore-limb buds are between 0.5 and 1 millimeter long. The larvae at this stage still lie on their sides for the most part. The ability to respond to the stimulus of light appeared rather suddenly in all. Six of the normal and five of the blinded larvae gave responses one morning, the rest were indifferent. In the afternoon of the same day they all responded to the light, giving a reaction within five minutes. The afternoon before they had all been insensitive to light. The average reaction time was longer than in the case of older larvae, being here about two hundred and twenty seconds. The larvae at this early stage all responded positively, and by means of very quick swimming movements. Sometimes a very quick movement which placed the long axis of the body in the direction of the rays was the total response. But usually they swam vigorously in the direction of the light stopping only when they came in contact with the side of the glass dish. Again, in a few cases some of them would swim vigorously for 6 or 7 cm. and then drop

passively to the bottom of the dish, always so oriented that their long axis was in the direction of the rays of light.

#### SUMMARY

1. Tadpoles of *R. pipiens* and of *R. sylvatica* show no response to the stimulus of light.
2. The larvae of *Amblystoma punctatum* are positively phototactic, both when normal and when deprived of their eyes.
3. The reactions of eyeless individuals are not brought about by stimulation of the central nervous system, but of the nerve terminals in the skin.
4. Normal larvae placed in light become pale, blinded larvae dark. Normal larvae placed in darkness become dark, blinded larvae pale, the conditions in the normal and blinded larvae thus being opposite.
5. The condition of the pigment in the skin chromatophores does not affect the sensitiveness of the larvae to light; but previous exposure to light, or adaptation to darkness, does, in that dark-adapted larvae are more sensitive to light than are those which have been kept in the light.
6. The youngest larvae to give responses to light of 768 candle-meters intensity were between 11 and 12 mm. long.

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# EXPERIMENTS ON THE REPRODUCTION OF THE HYPOTRICHOUS INFUSORIA

## II. A STUDY OF THE SO-CALLED LIFE CYCLE IN OXYTRICHA FALLAX AND PLEUOTRICHA LANCEOLATA

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SIXTEEN FIGURES (ONE PLATE)

### CONTENTS

I. Introduction.....	211
II. Methods.....	213
III. History of the cultures.....	214
1. Oxytricha fallax.....	214
a. Culture Ob.....	214
b. Culture Oh.....	215
c. Culture Ove.....	215
d. Culture Obt.....	217
2. Pleurotricha lanceolata.....	219
a. Culture Ph.....	219
b. Culture Pb.....	220
c. Culture Phlt.....	220
IV. Prepared material.....	223
1. Oxytricha fallax.....	223
2. Pleurotricha lanceolata.....	224
V. Discussion and conclusions.....	225
VI. General summary.....	230
Literature cited.....	233

### I. INTRODUCTION

As is well known, the early experimental work with the Infusoria gave evidence which was interpreted as supporting the view that these unicellular forms possess a definite life cycle comprising the generations between two successive conjugations, and on this account they can pass through but a certain number of generations, in the absence of conjugation, without senescence and death occurring. Calkins' work ('02) showed that

this so-called life cycle could be prolonged by 'artificial stimulation,' though he believed the cycle was real and that the stimuli used had only temporarily sustained the life of the culture in the absence of conjugation. Woodruff's work ('12 b) with *Paramecium aurelia* has shown that, given suitable conditions, this form can be bred indefinitely and the so-called life cycle entirely eliminated. In his work with *Paramecium caudatum*, the same investigator has shown that what appeared to be a typical life cycle in this organism was merely the dying out of the race due to unfavorable culture conditions. In Part I of these studies the writer has shown that fundamental changes can be brought about in the life history of the descendants from the same original animal by keeping the progeny on different culture media.

It appears, then, that the environment plays an important part in determining the length of the so-called life cycle and the character of the life history in the Infusoria. Inasmuch as the experimental work on the hypotrichous Infusoria has given evidence that these animals have a definite life cycle, it was decided to re-investigate the problem and endeavor to discover culture conditions in which these forms will live indefinitely without conjugation or artificial stimulation and thus eliminate the so-called life cycle for these hypotrichida as has been done by Woodruff with *Paramecium*. Accordingly, the experiments recorded in this paper were undertaken with the view of finding out the life history of the organisms studied when kept on different media and under varying culture conditions. In order to accomplish this, a number of cultures containing descendants of the same original individual were run at the same time under various culture conditions and consequently the life history of these cultures gives direct evidence on the effect of these varying conditions on the 'same protoplasm.' It was hoped in beginning the work that the conditions encountered, in some culture or cultures of the organisms studied, would be such as to permit the indefinite existence of the animals, and that the 'life cycle' might be eliminated in these forms. Also, if 'sister cells' kept under other culture conditions, which to begin with appeared to be entirely suitable, died out after passing through what ap-

peared to be a typical 'life cycle,' it would then be evident that they had not died through the ending of a definite life cycle, due to an inherent condition of senescence, but because the culture conditions had not been entirely suitable, inasmuch as 'sister cells' under other culture conditions were able to live indefinitely. In one of the two species employed in this work, *Pleurotricha lanceolata*, these expectations have been realized. The experimental work embraces a study of the following points:

1. The life history of *Oxytricha fallax* when bred in beef extract, hay infusion and 'varied environment' media under the conditions of daily isolation cultures.
2. The life history of the same organism when bred in a beef extract medium in mass cultures in test tubes.
3. The life history of *Pleurotricha lanceolata* when bred in beef extract and hay infusion media under the conditions of daily isolation cultures.
4. A study of a mass culture of *Pleurotricha lanceolata* kept on a hay infusion medium in test tubes.

These experiments have been carried on during a period of two years in the following laboratories: Sheffield Biological Laboratory and Osborn Zoological Laboratory of Yale University, Biological Laboratory of Central College, Pella, Iowa, and the Marine Biological Laboratory, Woods Hole, Massachusetts.

The writer desires to acknowledge his great obligation to Professor Lorande L. Woodruff for suggesting the problem and for the splendid assistance given during the course of the work, and also to Professor Alexander Petrunkevitch for assistance in making the microphotographs.

## II. METHODS

In Part I of these studies is given a full description of the methods used in carrying on the daily isolation cultures, the making of the beef extract, hay infusion and 'varied environment' media, the preparation of permanent specimens, and so forth. Inasmuch as all of these methods have been used in the present paper without modification, the reader is referred to the previous paper for these details.

### III. HISTORY OF THE CULTURES

1. *Oxytricha fallax*

The culture work with this hypotrichous infusorian was begun on October 19, 1911, by the isolation of a single specimen from a laboratory hay infusion. The individual was placed on a depression slide and some of the regular beef extract medium added. The progeny from this individual furnished the material for all the cultures of *Oxytricha fallax* mentioned in this paper. Four different cultures were under observation and each of these is described in detail in the following sections.

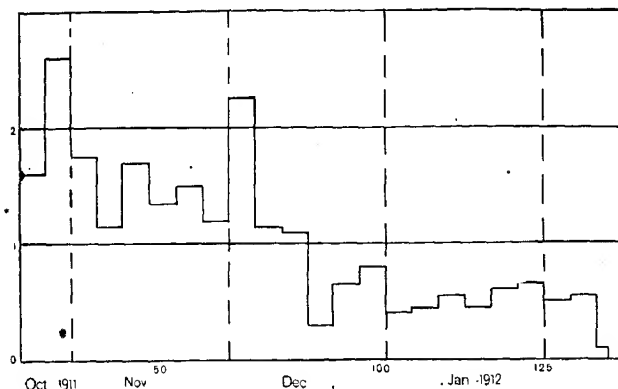


Diagram 1 Graph giving the life history of *Oxytricha fallax*, Culture Ob, showing the average daily rate of division of the four lines of the culture, again averaged for five-day periods. Beef extract culture medium.

*a. Culture Ob.* This daily isolation culture, bred on the bee medium, was started October 19, 1911, and continued until February 10, 1912, a period of 115 days, during which time it passed through 131 generations. Diagram 1 shows graphically the life history of this culture. With the exception of two five-day periods, the division rate was at all times below two divisions per day, and during the last two months the rate was never as

high as one division per day when averaged for five-day periods. Although the division rate was low, the animals appeared to be normal and healthy during the first three months of the culture, but about this time abnormal individuals began to appear and subsequently the culture died.

*b. Culture Oh.* This daily isolation culture, bred on the hay infusion medium, was started October 27, 1911, by isolating an individual from each of the four lines of the Ob culture, and was carried until February 8, 1912, a period of 105 days, during which time it passed through 159 generations. Diagram 2 shows graphically the life history. A study of the graph and a comparison

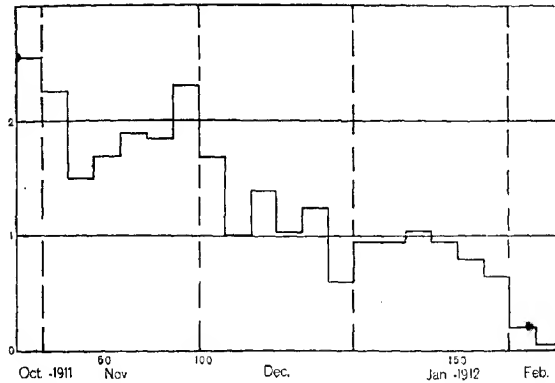


Diagram 2 Graph giving the life history of *Oxytricha fallax*, Culture Oh, showing the average daily rate of division of the four lines of the culture, again averaged for five-day periods. Hay infusion culture medium.

with diagram 1 shows that the life histories of these two cultures were much the same. This culture maintained a slightly higher division rate than did the Ob culture but died a few days sooner.

*c. Culture Ove.* This daily isolation culture, bred on the 'varied environment' medium, was started October 27, 1911, by isolating an individual from each of the four lines of the Ob

culture, and was carried until January 14, 1912, a period of 80 days, during which time it passed through 150 generations. Diagram 3 shows graphically the life history. Although this culture did not live so long as either of the other cultures it maintained a considerably higher rate of division. However, the decline which began about the middle of December continued quite rapidly and steadily and the culture died, as noted above.

It is observed, then, that, in all three of the daily isolation cultures of *Oxytricha fallax*, there is a general decline in the

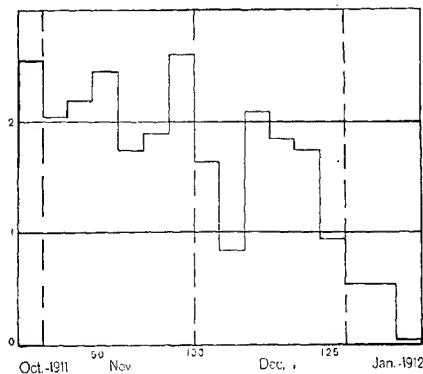


Diagram 3 Graph giving the life history of *Oxytricha fallax*, Culture Ove, showing the average daily rate of division of the four lines of the culture, again averaged for five-day periods. 'Varied environment' culture medium.

division rate almost from the time the cultures were first started. Of the three media used it appears that the 'varied environment' medium was to a slight degree less suitable for this animal than either the beef or the hay media. The animals of the Ove culture died out almost a month previous to those of the other two cultures. The rate of division was lower in the Ob culture than in either of the others but on the other hand this culture lived the longest. The highest number of generations was attained by the Oh culture. In brief, while some minor differ-

ences were evident in the life history of these three cultures in the way of division rate and length of time which they lived, they all showed what might be termed typical 'life cycles' ending with the death of the organisms.

*d. Culture Obtt.* A few days after the starting of the Ob culture, October 22, 1911, the animals from one of the stock slides were placed in a test tube containing some of the beef extract medium. This proved to be a favorable environment for the animals and the culture was continued by the following methods: The sterile beef extract medium is kept in test tubes, about 25 cc. in each. Into such a tube is introduced a little culture medium containing some of the animals. The animals thus introduced multiply very rapidly, if the conditions are favorable, and in about four or five days the maximum number is usually present. After this time the number of animals present gradually decreases, due chiefly, it is supposed, to a lack of sufficient food supply. When about the maximum number of animals is present, a little of the medium containing some of them is introduced into a fresh tube of the beef extract medium and thus the culture is continued. The bacteria which are introduced at the time of inoculation also multiply very rapidly in the fresh medium and furnish the food supply for the animals of the culture. The test tubes containing the animals are examined with great care daily by means of a Zeiss binocular microscope. By the use of such an instrument it is quite easy to examine the individuals contained in a small test tube very thoroughly and to be certain that conjugation is not occurring.

This mass culture in the test tubes was continued on the beef medium from October 22, 1911, until May 15, 1912. It was noted at this time that the animals in the culture were not multiplying as rapidly as usual and it was thought that a change of medium might be beneficial. Accordingly a large number of media of different kinds and varying strengths were made up from materials gathered from ponds and also from hay and grasses taken from different localities. These infusions were boiled to prevent contamination of the culture by foreign species



of Infusoria which might be present, and placed in test tubes. On most of these new infusions the animals thrived very well and were present in large numbers in the test tubes a few days later. Some of the animals were also kept as before on the beef medium in the test tubes but these all died by May 20. The other animals which had been transferred to the new media from the beef medium showed for a time a great improvement and appeared to have recovered their normal division rate but, about three weeks later, they again showed a low division rate and other signs of degeneration and this time the culture died out (June 10, 1912) after having been carried 231 days or almost eight months. It has been shown, as mentioned above, that the maximum time that these animals lived, in any of the daily isolation cultures, was 112 days. We have, then, under the conditions in the mass culture more than a doubling of the length of the time of the so-called life cycle as found in the daily isolation cultures.

To summarize briefly the culture work with this race of *Oxytricha fallax*: Three daily isolation cultures and one mass culture in test tubes were carried, namely, Culture Ob, a daily isolation culture which attained 131 generations in 115 days on a beef extract medium; Culture Oh, a daily isolation culture which attained 159 generations in 105 days on a hay infusion medium; Culture Ove, a daily isolation culture which attained 150 generations in 80 days on a 'varied environment' medium; Culture Obtt, a mass culture kept in test tubes which lived 231 days on the beef extract medium. It is obvious from the experiments with this race of *Oxytricha fallax* that, although it was impossible to prolong the life of the race indefinitely by any of the media employed, nevertheless it was possible, by the use of the test tube method to greatly extend the so-called life cycle. This result supplies additional evidence that the length of the 'life cycle' and the character of the culture methods are intimately related.

2. *Pleurotricha lanceolata*

The culture work with this hypotrichous infusorian was begun on January 6, 1912, by the isolation of a single specimen from a laboratory hay infusion. This individual was placed on a depression slide and some of the regular hay infusion added. The species proved to be one that was well adapted for experimental work of this kind, and the progeny from this individual furnished

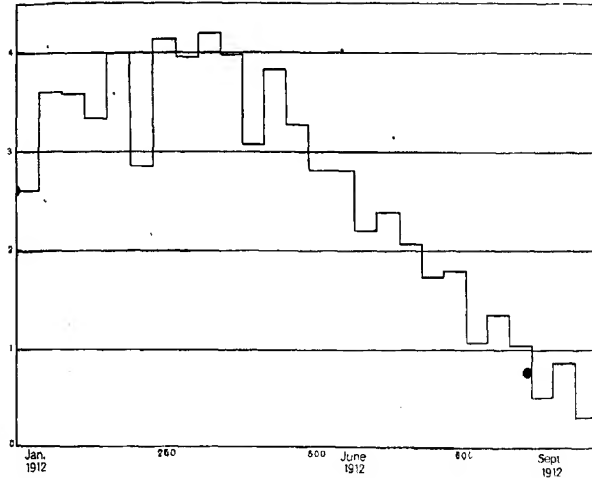


Diagram 4 Graph giving the life history of *Pleurotricha lanceolata*, Culture Ph, showing the average daily rate of division of the four lines of the culture, again averaged for ten-day periods. Hay infusion culture medium.

the material for all the cultures of *Pleurotricha lanceolata* mentioned in this paper. Three different cultures were under observation and each of these is described in detail in the following sections.

*a. Culture Ph.* This daily isolation culture, kept on the hay medium, was started January 9, 1912, and continued until September 25, 1912, a period of 259 days, during which time it

passed through 656 generations. Diagram 4 shows graphically the life history of this culture. At the time the culture was started the animals were dividing at an average rate of 2.5 divisions in twenty-four hours. This rate gradually increased for a time as shown in the graph until the culture reached the remarkably high rate of four divisions per day. The records show that an average rate of about four divisions per day was maintained for forty days. However, even though the division rate was very high and the animals of the culture gave every evidence, so far as could be determined, of being in an environment which was suited to their needs, a decline in the rate began at the end of the forty-day period mentioned above and this decline steadily continued and finally resulted in the death of the culture (September 25, 1912).

*b. Culture Pb.* This daily isolation culture kept on the beef medium, was started January 9, 1912, and carried until May 1, 1913, a period of 478 days, during which time the culture passed through 943 generations. Diagram 5 shows graphically the life history of this culture. As will be noted in the graph, a division rate averaging about 2.5 divisions in twenty-four hours was maintained for the first twenty periods (200 days). The decline which began at this point continued very slowly but steadily, the rate finally falling below two divisions in twenty-four hours and during the last two months of the life of the culture the rate was never as high as one division per day when averaged for a ten-day period. The culture finally died out at the 943d generation on May 1, 1913.

In both of these daily isolation cultures of *Pleurotricha lanceolata*, a study of the graphs reveals what could be termed typical 'life cycles.' When once the decline began with few exceptions it steadily continued and the maximum of each succeeding rhythm generally was lower than the maximum of the preceding one.

*c. Culture Phll.* This mass culture kept on a hay infusion medium in test tubes was started January 9, 1912, at the same time that the two daily isolation cultures (Ph and Pb) were started and has been carried continuously since then up to the present

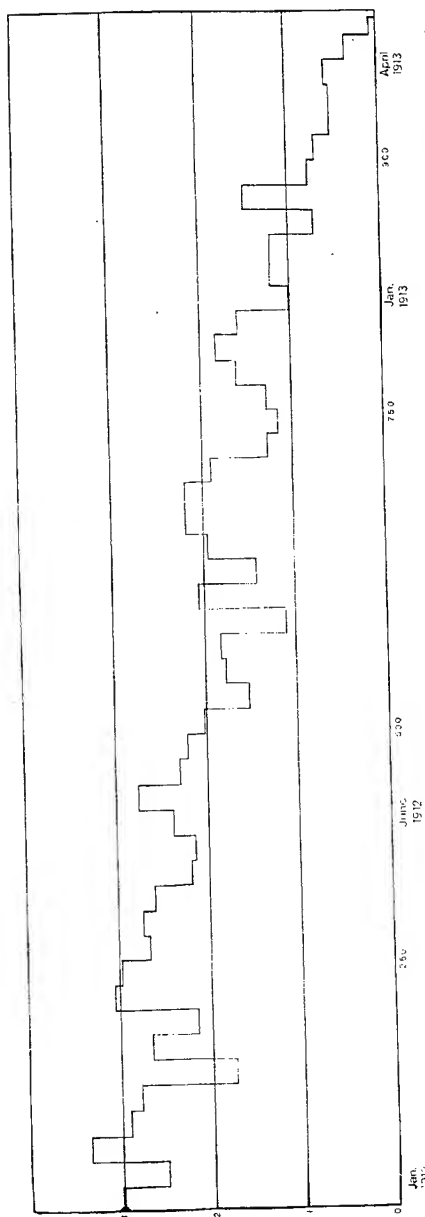


Diagram 5. Graph giving the life history of *Pleurofridula lanceolata*, Culture Pb, showing the average daily rate of division of the four lines of the culture, again averaged for ten-day periods. Beef extract culture medium.

time (November 20, 1913), a period of more than twenty-two months, under these same conditions.

The method of carrying a test tube culture has already been described above (see Culture Obtt, p. 217) and this same method has been used in this case, except that the medium used has been hay infusion instead of the beef medium.

At any time during the period that the culture has been under observation it has been possible to secure almost any number of animals simply by inoculating a suitable medium with a few of them from the regular test tube culture. Great numbers of the animals obtained in this way have been used in class work, for the study of both living and prepared material, during the course of the experiments, and no abnormal specimens have ever been found. In order to test the division rate of the animals from this culture after they had been subjected to the culture conditions described above for almost twenty months, a daily isolation culture on the hay infusion medium was started on July 8, 1913, and continued for a period of 35 days. In starting it, one individual was taken from this test tube culture and placed on a depression slide. During the night this individual divided twice, forming four animals, and these were isolated and placed on depression slides to start the four lines of the daily isolation culture. During the 35 days that this culture was under observation, the animals maintained a division rate of almost exactly 2.5 divisions per day. It is evident from this experiment that the animals of this culture are capable of maintaining as high a division rate now as when the culture was first started.

The animals of this test tube culture have given at all times during the more than 22 months that they have been under observation every evidence of being in an environment which is entirely suitable for them and *it is believed that these animals can be bred indefinitely under these conditions without conjugation or artificial stimulation of any kind.*

To summarize briefly the culture work with this race of *Pleurotricha lanceolata*: Two daily isolation cultures and one mass culture in test tubes were carried, namely: Culture Ph, a daily isolation culture which attained 656 generations in a period of 259

days on a hay infusion medium; Culture Pb, a daily isolation culture which attained 943 generations in a period of 478 days on a beef extract medium; Culture Phtt, a mass culture kept in test tubes on the hay infusion medium, which is still thriving at the present time (November 20, 1913) more than 22 months after it was first started and which gives every evidence of being able to live indefinitely under these conditions without conjugation or artificial stimulation.

#### IV. PREPARED MATERIAL

The study of the prepared material of *Oxytricha fallax* and *Pleurotricha lanceolata*, taken from the cultures which died out, reveals the fact that unsuitable culture conditions may bring about great nuclear and cytoplasmic changes in these organisms.

##### 1. *Oxytricha fallax*

Figures 1 to 6 are from material taken from the *Oxytricha fallax* cultures. The animals preserved at the beginning of the work showed a normal morphological condition, as is seen, for example, in figure 1. Among the individuals preserved a month or so later occasionally an abnormal individual was found. An example of this is shown in figure 2. The cytoplasm of this animal still appears to be in a practically normal morphological condition but in the nuclear structure is to be seen evidence of degenerative changes. As the cultures became still older a very much greater proportion of atypical individuals were found among the specimens preserved and in many cases the appearance of the animals while still alive showed that they were not normal. Shortly before the death of the daily isolation cultures practically every animal that was preserved showed a degenerate condition. In figures 3 and 4, which are from specimens preserved later in the work, are shown other specimens of degenerate animals. Figures 1 to 4 were all taken from specimens preserved from the daily isolation cultures.

The animals mentioned above apparently do not differ from those obtained by former investigators along this line and by

whom the degeneration was thought to be due to an inherent condition of senescence. In these experiments, it can be said that such was not the case, for 'sister cells' which were kept under other conditions in the mass test tube culture were, as far as could be told both by careful observation of the living animals and a thorough study of the prepared specimens, normal at the time when, and for some time after, the daily isolation cultures had died out. In figure 5 is shown a typical example of the animals present in the test tube culture at this time. This specimen was preserved February 15, which was several days after the last of the daily isolation cultures had died. Figure 6 is a specimen preserved from the test tube culture May 18 and it shows an atypical condition of the nucleus and cytoplasm. Before this time specimens preserved from this culture gave every evidence of being in as normal condition as is shown in figure 5. After this time, however, the number of abnormal animals in the test tube culture rapidly increased, due, it is believed, as in the daily isolation cultures of this organism, to the cumulative effects of an environment not entirely adapted to the organism studied, and subsequently (June 10, 1912) this culture died.

## 2. *Pleurotricha lanceolata*

Figures 7 to 11 are from *Pleurotricha lanceolata* cultures. In figure 7 is shown a typical normal individual. This specimen was taken from the Ph culture soon after it was started. Figure 8 shows an animal taken from the same culture several months later shortly before it died out. Another atypical specimen is seen in figure 9 which was taken from the Pb culture at about the same time. As in the *Oxytricha fallax* cultures so also here in the *Pleurotricha lanceolata* cultures the changes which appeared in the animals in the cultures that died out is shown not to be due to 'senile degeneration' inasmuch as 'sister cells' which were kept under other culture conditions do not show simultaneous degenerative changes. Figure 10 is a typical normal specimen preserved from the Ph<sub>tt</sub> culture some time after

the death of the Ph culture, and in figure 11 is shown another normal individual from the same culture preserved October 20, 1913, *about 13 months after the death of the Ph culture and 6 months after the death of the Pb culture.* As has been noted above, *no abnormal specimens have ever been found in the Phlt culture during its entire history, covering, thus far, a period of over 22 months.*

#### V. DISCUSSION AND CONCLUSIONS

A number of investigators have noted the fact, as shown in these experiments, that closely related species of the Infusoria, or even different races of the same species, vary greatly in regard to culture conditions that are adapted to them. Jennings and Hargitt ('10), working on a number of different races of paramaecia, found that culture conditions which were apparently ideal for a certain race of *Paramecium* might not be at all suitable for another race. Woodruff ('11) found that culture conditions which were entirely suitable for his *Paramecium aurelia* cultures and on which they had thrived for years were not entirely adapted for a race of *Paramecium caudatum*. The experiments of Woodruff ('12), in his researches on the protozoan fauna of hay infusions, indicate that the animals are adapted for only certain conditions of the medium, and when these conditions are attained, they are present in maximum numbers, and as the conditions in the infusion change they disappear and other forms become dominant which are adapted to the new conditions.

In Part I of these studies, the writer ('12) was able to show that it is possible to change greatly the life history of *Stylonichia pustulata* by employing different kinds of culture media. In a culture bred on beef extract medium conjugation occurred, whereas, in another culture of descendants from the same original animal but kept on a hay infusion medium, conjugation did not occur at any time. The animals in the beef culture died at the 403d generation while those on the hay medium attained 572 generations. The experiment was repeated by starting another culture on the beef extract medium with animals from the hay culture after the first beef culture had died, and again conjugation



tion occurred in this second beef culture. Inasmuch as the only difference in the treatment of the two cultures lay in the media used, there can be no doubt that the fundamental changes in the life histories of the hay and beef cultures were induced by the culture media. To quote from the former paper (p. 71):

Inasmuch as conjugation never occurred at any time in the hay culture, even though in both the length of time it was kept and the number of generations through which it passed it exceeded either of the cultures kept on the beef medium, there is conclusive evidence that neither the age of the organisms nor the number of generations through which they passed were potent in inducing conjugation in these cultures. *The determining feature was the medium used and the results here recorded give definite evidence that the 'same protoplasm' under the influence of different culture media may show fundamental differences in its life history.*

Calkins ('13), in some recent experiments on *Paramecium*, isolated the four cells resulting from the first two divisions of an ex-conjugant and, later, when each of these four cells had divided three times, forming eight cells, or 32 cells in all, he again isolated them, forming four 'quadrants' of eight lines each which were continued as pure lines. During the time that the animals have been under observation (about seven months) conjugation has been found to occur in only one of the 'quadrants.' Calkins concludes from these results, but more particularly from the fact that no conjugation has occurred in Woodruff's *paramecium* cultures during their long period under observation,<sup>1</sup> that "some descendants of an ex-conjugant are potential germ cells, others are not" or, in other words, that in *paramecia* there are conjugating and non-conjugating races, due to an inherent difference in the four cells derived from a single ex-conjugant. However, Calkins in the preceding volume of this journal presents the results in a complete form. These show that conjugants have now been found in *all* the 'quadrants' of the original *J* series. To quote from page 448: "The April test brought a surprise" in that animals from 'quadrants' *B*, *C* and *D* all gave some conjugating pairs, as they also did in a second test later

<sup>1</sup>For later results see article by Dr. Woodruff in this number.

in April, so that "all four quadrants are now represented in the conjugating lines." Inasmuch, then, as all the 'quadrants' are now shown by these later results to possess the power of conjugation, the results of the experiments do not seem to be such as to prove that there are conjugating and non-conjugating races present in these cultures. The explanation as to why some of the 'quadrants' conjugate more freely than others may lie in some external condition or conditions of the environment rather than in any inherent difference in the animals of the various 'quadrants,' since, as noted above, my work with *Stylonychia pustulata* has shown that a 'conjugating race' and a 'non-conjugating race,' using Calkins' terms, may be obtained from the descendants of a single individual not an *ex-conjugant* by the subjection of the different lines to various culture conditions.

In the experiments recorded in the present paper it has been shown that *Oxytricha fallax* under certain culture conditions, which at the beginning of the work appeared to be entirely suitable, lived about three and one-half months, but that under other culture conditions they lived nearly eight months, or more than twice as long. In *Pleurotricha lanceolata*, also, the ability of different culture conditions to vary the life history of the organism has been clearly shown in that one of the cultures lived 259 days, another lived 478 days, and another gives every promise, after over 22 months of existence, of having culture conditions which are entirely suitable and in which it can live indefinitely without conjugation or artificial stimulation.<sup>2</sup>

The graphs showing the division rate of all the daily isolation cultures, of both the organisms studied, exhibit what may be

<sup>2</sup> I cannot agree with the position of Calkins and Gregory ('13) which appears to be essentially that in order to 'prove' the 'immortality' of the Protozoa they must be bred through eternity. The generally accepted work of Woodruff with a race of *Paramecium aurelia* has established beyond the question of a doubt, I believe, that in this form at least an indefinite and unlimited multiplication without conjugation or artificial stimulation may be had, provided suitable culture conditions are supplied. The experiments in these studies show that the same holds true for at least one of the hypotrichous Infusoria and also that the so-called life cycle may be greatly and variously modified, depending upon the conditions in which the animals are bred.

termed 'typical life cycle' curves. Also the study of the prepared material, from the cultures of both species which died out, reveals morphological and nuclear changes which can be interpreted as being due to 'senile degeneration.' However, since, as has been noted above, 'sister cells' from the test tube cultures of *Oxytricha fallax*, preserved at the same time or even later than cells in a degenerate condition taken from the daily isolation cultures and which should therefore show at least as great a degeneration, reveal a normal nuclear and cytoplasmic condition and, also, because of the fact that in *Pleurotricha lanceolata* no degenerate or abnormal individuals have ever been found in the test tube culture during the entire period that it has been under observation, it is evident that the atypical conditions that were present in the cells of the cultures which died cannot be attributed to an inherent condition of senescence. The results obtained clearly indicate that the degenerative changes which occurred and the subsequent dying out of the cultures resulted not from a condition of 'senile degeneration' in the animals, but from the fact that the culture conditions supplied in these cases were not entirely suitable, and consequently, it is believed, that the length of the so-called life cycle in these hypotrichous infusorian forms is not a question of an inherent ability to attain only a certain number of generations, but it is dependent upon the degree to which the culture conditions are favorable. *If the conditions supplied are entirely adapted to the needs of the organism, as in the test tube culture of Pleurotricha lanceolata, it seems evident that there will be no so-called life cycle and the organism will continue to multiply as long as these conditions are maintained.*

When the results of some of the earlier investigators which support the view that the Infusoria possess a definite life cycle ending at a certain number of generations are considered, it appears that these results have been due to the cumulative effects of culture conditions which were to some degree unsuitable to the organisms under observation and which, therefore, were not adapted to their indefinite existence.

The question of artificial stimulation as a means of prolonging the so-called life cycle of various protozoan forms should also

be mentioned. Calkins ('02), for example, has shown in his work with *Paramecium caudatum* that, at times, a decided change of medium may result in a 'rejuvenation' and the animals which before had been dividing very slowly and showing other signs of degeneration, will take on a new lease of life and appear normal for a time. This 'artificial rejuvenation' could be accomplished a number of times but finally the organism reached a stage where no 'rejuvenation' resulted from any of the methods tried and the animals of the culture died out. Woodruff ('05) also found in his work with one of his cultures of *Oxytricha fallax* that these animals were 'artificially rejuvenated' by the use of a beef extract medium. At about the 300th generation, after the culture had been under observation for about ten months, the animals of the culture were dividing very slowly and it seemed that they would all die, when they were 'rejuvenated' by the use of the beef extract and finally reached the 860th generation, almost a year later, before the culture died out. At the time it was believed that the degeneration, which appeared in the animals of the cultures under observation, was due to an inherent condition of senescence, but it would appear, from the more recent researches, that the degeneration resulted from the cumulative effects of culture media or culture conditions which were not entirely adapted to the organisms in question. Woodruff in a recent paper ('13) holds this view with regard to his culture of *Oxytricha fallax* and bases his conclusion upon the results of his work with *Paramecium aurelia*. To quote (p. 8):

Therefore in the light of studies during the past decade, I would interpret the cycle of my *Oxytricha A* culture as being the result of the fact that the race of *Oxytricha fallax* which was employed was not adapted to live indefinitely 'when continuously subjected to the same environment' of hay infusion ('05, p. 627). I believe that if an entirely suitable environment had been secured this culture would have given evidence of unlimited power of reproduction by division without conjugation as my present *P. aurelia* race has done.

In the cases of 'artificial rejuvenation,' it seems evident, then, that the change of medium brought about a temporary relief

from unsuitable culture conditions but since none of the media supplied happened to be entirely suited to the needs of the organism, death invariably resulted after a time.

From the results of these experimental studies mentioned above it is believed that only one conclusion can be drawn and that is that *the length of the so-called life cycle in the hypotrichous Infusoria, as in Paramecium, is not a question of senescence or, in other words, a tendency of the protoplasm to grow old, but it is dependent upon whether or not the organisms are supplied with an environment which is adapted to their particular requirements.* Given a species which is adapted to the culture conditions in which it is bred there is reason for believing that it can be bred indefinitely without the necessity of conjugation or of artificial stimulation. If the culture conditions supplied are unfavorable to the organism, marked nuclear and cytoplasmic degenerative changes occur and the death of the culture inevitably results, sooner or later, depending upon the degree to which the conditions are unfavorable, the natural resistance of the species, and its ability to adapt itself to the new conditions.

#### VI. GENERAL SUMMARY

1. The object of these experiments was (1) to determine if culture conditions could be supplied to the hypotrichous Infusoria which would eliminate the so-called life cycle in these forms, and (2) to observe the effect, upon the life history of the organisms studied, of the various culture conditions supplied.

2. Three media were used in these experiments, namely, a 'constant' medium consisting of a 0.025 per cent solution of Liebig's extract of meat, a hay infusion medium, and a 'varied environment' medium. Both daily isolation cultures and mass cultures kept in test tubes were employed in this work.

3. Four cultures of *Oxytricha fallax* have been under observation, all of which were started from descendants of an original individual isolated from a laboratory hay infusion, October 19, 1911. Culture Ob was started October 19, 1911, and was carried until February 10, 1912, on a beef extract medium, when it died

out at the 131st generation. Culture Oh was started October 27, 1911, by isolating an individual from the Ob culture, and it was carried until February 1, 1912, on a hay infusion medium, when it died out at the 159th generation. Culture Ove was started October 27, 1911, by the isolation of an individual from the Ob culture and it was carried until January 14, 1912, on a 'varied environment' medium, when it died out at the 150th generation. These three were all daily isolation cultures. Culture Obt, a mass culture, carried on a beef extract medium and kept in test tubes, was started October 22, 1911, by the isolation of an individual from the Ob culture and was carried until May 15, 1913, when it died out. Graphs showing the life history of each of the daily isolation cultures have been plotted by averaging the divisions in the four lines of each culture for five-day periods.

4. Three cultures of *Pleurotricha lanceolata* have been under observation, all of which were started from descendants of an original individual isolated from a laboratory hay infusion January 6, 1912. Culture Ph was started January 9, 1912, and was carried on a hay infusion medium until September 25, 1912, when it died out at the 656th generation. Culture Pb was started January 9, 1912, and was carried until May 1, 1913, on a beef extract medium, when it died out at the 943d generation. Both of these were daily isolation cultures. Culture Phtt, a mass culture kept in test tubes on a hay infusion medium, was started January 9, 1912, and has, so far, been carried over 22 months and gives every evidence of being able to live indefinitely. Graphs showing the life history of each of the daily isolation cultures have been plotted by averaging the divisions in the four lines of each culture for ten-day periods.

5. The study of the graphs in all the daily isolation cultures of both species reveals what might be termed typical 'life cycles' in these organisms.

6. The study of the prepared material shows that, although the animals at the beginning of the work gave every evidence of a normal morphological condition, previous to the death of a culture atypical and degenerate individuals appear and these

are similar to degenerate animals found by former investigators, by whom they were thought to be due to an inherent condition of senescence in the animals.

7. In these experiments the results indicate that the dying out of some of the cultures was due, not to a condition of inherent senescence, but to the fact that the culture conditions supplied were not entirely favorable to the indefinite existence of the organisms under observation, since in *Oxytricha fallax* 'sister cells' bred in a mass test tube culture lived more than twice as long as those bred in daily isolation cultures, and in *Pleurotricha lanceolata* culture conditions have been found in which this organism *will apparently live indefinitely without conjugation or artificial stimulation.*

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## PLATE 1

### EXPLANATION OF FIGURES

These photographs were taken from permanent preparations stained with picrocarmin. The same magnification (600 diameters) was used in all cases and, therefore, the relative sizes represent absolute differences in the size of the different individuals.

#### *Oxytricha fallax*

1 A typical normal individual taken from the Oh culture at the beginning of the work. One of the micronuclei has divided prior to cell division.

2 to 4 In these figures are to be seen types of the abnormal animals which appeared later in the history of the cultures. Figure 2 was taken from the Ove culture, December 12, 1911, at the 110th generation. Figure 3 was taken from the Oh culture January 27, 1912, at the 158th generation. Figure 4 was taken from the Ob culture January 27, 1912, at the 121st generation.

5 A typical normal individual taken from the Obtt culture on February 15, 1912, several days after the death of the last of the daily isolation cultures.

6 An atypical individual taken from the Obtt culture on May 18, 1912, about two weeks before this culture died out.

#### *Pleurotricha lanceolata*

7 A typical normal individual taken from the Ph culture at the beginning of the work.

8 An atypical individual taken from the Ph culture August 28, 1912, at the 637th generation. This culture died out four weeks later.

9 An atypical individual taken from the Pb culture August 28, 1912, at the 597th generation.

10 A normal individual taken from the Phtt culture September 7, 1912.

11 A normal individual taken from the Phtt culture October 20, 1913.

REPRODUCTION OF THE HYPOTRICHIOUS INFUSORIA  
GEORGE ALFRED BAITSELL

PLATE I





## SO-CALLED CONJUGATING AND NON-CONJUGATING RACES OF PARAMAECIUM

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ONE FIGURE

An interesting characteristic of the pedigreed race of *Paramecium aurelia*,<sup>1</sup> which I have had under daily observation for the past six years and eight months, has been the apparent abeyance of the tendency to conjugate during the first seventy-nine months of its existence. Although the possibility of conjugation has been prevented in the main lines of the race, a large series of experiments have been made from time to time to induce conjugation in mass cultures started with individuals left over from the daily isolations from the main lines. These experiments apparently failed to afford just the proper environmental conditions to initiate conjugation<sup>2</sup> for not a single pair had been observed before the experiment to be described below.

On December 1, 1913, a large jarful of water and decaying plants from an old laboratory amoeba culture was thoroughly boiled for half-an-hour and a part of this material was then put into a large, sterilized stender dish with ground-on cover, while the rest was left in the container in which it was boiled. The following day the contents of the stender dish was inoculated with a considerable number of paramaecia left over from the isolations that day (4102d generation).<sup>3</sup> At the end of five days there was a heavy pure growth of paramaecia in the stender dish but no protozoa of any kind in the uninoculated culture material.

<sup>1</sup> For details of the race, cf. L. L. Woodruff: *Archiv für Protistenkunde*, Bd. 21, 1911; *Biochemical Bulletin*, vol. 1, 1912.

<sup>2</sup> E.g., Baitsell (*Jour. Exp. Zool.*, vol. 13, 1912, p. 73) from his experiments on the life history of *Stylonychia* shows "that conjugation is induced by external conditions affecting the organism."

<sup>3</sup> Cf. figure 1.

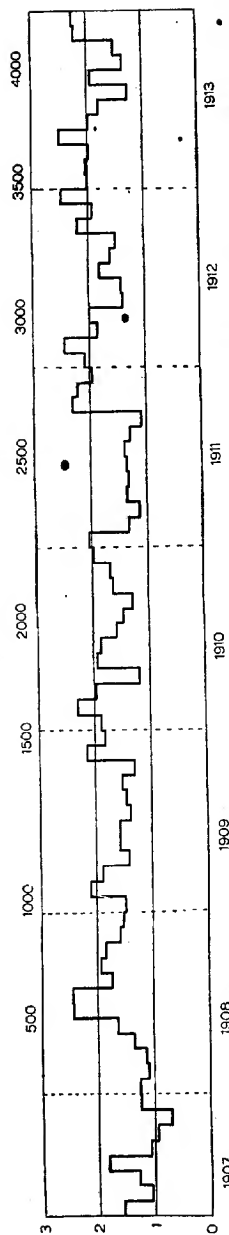


Fig. 1 Graph showing the rate of reproduction of the pedigreed race (I) of *Paramaecium aurelia* from start on May 1, 1907, to the present time, January 1, 1914, at the 4167th generation. The ordinates represent the average daily rate of division of the four lines of the culture again averaged for each *month* of its life to date. The vertical broken lines indicate the limits of the calendar years. The figures 500, 1000, etc., represent generations attained at the various periods.

Eight depression slides were each filled with about five drops of this culture of *Paramecium* and placed in a moist chamber at room temperature. The following morning (December 7, 1913) a couple of conjugating pairs were discovered on each slide. At this time material from the stender dish was again distributed on depression slides and the next day again a couple of pairs were isolated from several of these slides. About twenty pairs were thus secured, some of which have been preserved at various stages for cytological study and others as exconjugants initiated lines which are now being bred by the same pedigreed culture methods employed for the race from which they were originally derived. Details of this work will be presented in a later paper.

During the more than six and one-half years of the life of this race a considerable amount of evidence has been accumulated by different observers tending to show that the conditions determining conjugation vary greatly in different races and in different lines within the same race of *Paramecium aurelia* and *Paramecium caudatum*. Jennings<sup>4</sup> writes: "Some races conjugate frequently, and under conditions readily supplied in experimentation. Others, under the same conditions, conjugate very rarely or not at all." Calkins<sup>5</sup> finds that "Some lines will conjugate whenever the conditions favorable for conjugation are prepared; other lines have never conjugated under such conditions."

These variations in the tendency to conjugate which are exhibited by pure races and lines of *Paramecium* apparently have led Calkins to believe that "The traditional view that each *Paramecium* is a potential germ cell is not true,"<sup>6</sup> and that herein lies the clue to the directly opposite results derived from his races and from mine.<sup>7</sup>

<sup>4</sup> H. S. Jennings, Jour. Exp. Zool., vol. 9, 1910, p. 298.

<sup>5</sup> G. N. Calkins and L. H. Gregory, Jour. Exp. Zool., vol. 15, 1913, p. 509.

<sup>6</sup> G. N. Calkins, Proc. Soc. for Exp. Biol. and Med., vol. 10, 1913, p. 67.

<sup>7</sup> Calkins (loc. cit.): "The life history of conjugating lines has shown that if conjugation is prevented, the race dies out." Woodruff (loc. cit.): "I believe this culture shows clearly that *Paramecium aurelia* when subjected to suitable culture conditions, has the power of unlimited reproduction by division without conjugation or artificial stimulation."

The race that I worked with in 1901 was a conjugating race which died out in the 742d generation. Woodruff's long line of over 3500 generations is a non-conjugating race and the two races cannot be compared in regard to vitality, since normal conjugation was prevented in the conjugating race, whereas in the non-conjugating race there has been no artificial prevention of a normal process.<sup>8</sup>

In an extensive paper on physiological variations in *Paramecium*, Calkins and Gregory reiterate the same point of view:

To return to Woodruff's race of *Paramecium aurelia*, we find this important difference between his material and that which Calkins worked with. The *Paramecium caudatum* which formed the material for the earlier observations was a conjugating line as shown by conjugation tests made from time to time. In this line, therefore, a normal function—conjugation—was inhibited; metabolism weakened steadily until physiological death carried off all of the race not artificially stimulated. Finally, "germinal death" preceded by the degeneration of the micronucleus and the cortical plasm, carried off the last individual. . . . In Woodruff's material on the other hand, the race is non-conjugating, hence no normal function has been suppressed by keeping the individual apart. So far as known the life history of such non-conjugating forms has never before been undertaken and Woodruff has shown that they will live for at least five and one-half years.<sup>9</sup>

The fact that conjugation has now occurred in mass cultures from my pedigreed race demonstrates that the race is a conjugating race when the proper conditions for its consummation are realized. Therefore there is no evidence extant that a non-conjugating race of *Paramecium* exists. One who now would demonstrate its existence must plan to carry a race for far more than 4102 generations without a tendency to conjugation being manifested.

<sup>8</sup> G. N. Calkins, *Proc. Soc. for Exper. Biol. and Med.*, vol. 10, 1913, p. 67.

<sup>9</sup> Calkins and Gregory, *loc. cit.*

# THE SUPPOSED EXPERIMENTAL PRODUCTION OF HEMOLYMPH NODES AND ACCESSORY SPLEENS

## V. STUDIES ON HEMAL NODES

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For some decades it has been customary to speak of at least two types of lymph glands or nodes—the ordinary lymphatic gland and the hemolymph node. Although these two types of nodes have been said to occur in man (2, 3, 5, 11, 17, 20, 21, 28, 38), monkeys (17, 37, 37 a), rodents (18, 36, 44), most of the domestic animals (2, 3, 4, 5, 6, 11, 12, 18, 21, 29 a, 36, 37, 38, 39, 44, etc.), birds (18, 36, etc.), fish (36, 37 a), and so forth, those who studied them in greater detail soon found that neither gross nor microscopical differentiation was possible because all manner of transition or intermediate forms were thought to exist. Vincent and Harrison (42), for example, make the following statement:

We believe that we have found gradual transitions from hemolymph glands on the one hand, to ordinary lymphatic glands, and on the other hand, to the structure of the spleen. So that no hard and fast line can be drawn marking off these structures from one another. A lymphatic gland has only to contain blood in part or the whole of its sinuses to constitute itself one of the varieties of hemolymph glands. In certain 'accessory spleens' moreover, the splenic reticulum is so widened out as to approximate to a blood sinus of a hemolymph gland and the Malpighian bodies are so diffuse as to resemble the lymphoid portions of the blood-lymph glands. So that spleen, hemolymph glands and ordinary lymphatic glands form almost a continuous series.

A similar opinion was subsequently expressed by Warthin who further satisfied himself that hemolymph nodes can be produced experimentally and that there is a transition from fat to hemolymph nodes to lymph glands and back again to fat.



In spite of the confessed impossibility of distinguishing between lymph and hemolymph nodes Lewis and Warthin formulated rather elaborate classifications in which the lymphatico-hemal structures of the body were divided into lymphatic, hemal-lymphatic, and hemal nodes. According to Lewis, two types of hemal nodes with an intermediate form, and three types of hemal lymphatic glands can be distinguished. Warthin (46) suggested a further division in spleno- and marrow hemolymph nodes and Morandi and Sisto (25) divide human hemolymph nodes into six groups even. Weidenreich (51) also distinguished several types of hemolymph nodes but upon the basis of injections nevertheless concluded that hemolymph nodes as a class have no connection with the lymphatic system. The latter was, however, denied by Helly (19) and Forgeot (11) who claimed that hemolymph glands did not only contain lymphatics but that all manner of transition forms could be found in which lymphatic vessels simply were near the hilus, in the hilus, and in various stages of penetration of the node. A similar position is also taken by v. Schumacher (4) who concluded that a lymph node may become a hemolymph node and finally perhaps again a lymph node by the disappearance and perhaps the re-entrance of lymphatics. v. Schumacher also considered the hemal node as a rudimentary form of ordinary lymph nodes and divided the latter into those with and without lymphatics.

As will be reported elsewhere, a very large series of injections directly on the carcasses of sheep, has led to the positive conclusion that Weidenreich was correct when he decided as a result of similar injections that the true, not the so-called, hemolymph nodes have no connection with the lymphatic system. If this is true it follows, of course, that this is a simple and crucial test for differentiating lymph and hemolymph nodes although v. Schumacher denies the latter. By means of it the alleged production of hemolymph nodes under various conditions can also be tested easily. If, for example, certain *apparently* newly-formed nodes are found or if lymph nodes have apparently been transformed into hemolymph nodes, the identity of the former and the absence of lymphatic connections in the latter can readily

and positively be determined by means of the injection method. It is clear, of course, that no lymph node could become a true hemolymph (hemal) node without being deprived of its intrinsic lymphatic circulation and its connection with the lymphatic system. Furthermore, the vascular circulation of lymphatic nodes would also have to become altered very profoundly before it could even remotely simulate the vascular circulation of hemolymph (hemal) nodes, a fact wholly disregarded by v. Schumacher. Besides certain modifications in the cellular content would also have to occur.

It will be recalled that Tizzoni (35 and 36, et seq.), Eternod (7) and others described splenic nodules on the peritoneum and great omentum which they regarded as having formed as a result of physiological compensation after deficiency in function of the main spleen. Griffini (15) reported similar results and believed new spleens formed from the omentum of the main spleen but Foa (8 and 9) on the contrary regarded these so-called newly formed accessory spleens as normal variations the existence of which Tizzoni had overlooked. However, Warthin (48) referring to Tizzoni's experiments says that the latter's "description of their formation in adipose tissue is confirmed in every detail by the findings in the above cases. (Splenectomies.) His conclusion alone must be regarded as erroneous." Tizzoni's conclusion was that accessory spleens were formed *de novo* while Warthin concluded as Morandi and Sisto (25) had previously done, that the splenic nodules found by Tizzoni, Eternod, Griffini, Mosler and Winogradow were not accessory spleens at all, but hemolymph nodes present before operation but which Warthin considered as newly formed. The cases to which Warthin refers are splenectomies on goats and sheep which resulted in a "new formation of hemolymph nodes from adipose tissue and the transformation of hemolymph into ordinary lymphatic glands." This new formation of hemolymph nodes in fat was observed by Warthin within one month after splenectomy. Subsequently to these experiments Warthin<sup>1</sup> further reported the formation of

<sup>1</sup> Program of Am. Assoc. Path. and Bact.; 11th Annual Meeting. Chicago, 1911.

hemolymph nodes in a dog after splenectomy but I have unfortunately only been able to find the report by title. Vincent (43) upon examining a dog 16 months after splenectomy stated that "There was certainly no increase in the number or size of the ordinary lymphatic glands, but there were some half dozen (rather more than the usual number) of the intermediate form referred to above, i.e., which showed some signs, even to the naked eye, of containing blood." Nevertheless, in order to satisfy himself further regarding this matter Vincent performed splenectomies upon five dogs varying in age from approximately one to two years. From these animals which were killed from five to six months after operation, Vincent reported as follows: "In none of them could I be sure that there was any increase in size and number of the hemal lymphatic glands, nor could I assure myself that of those present any larger portion than usual was occupied by a blood-sinus instead of a lymph sinus." These observations of Vincent seem to confirm the *findings* though not the conclusions of Tizzoni (35 and 36). The latter found reddened lymph nodes in only two out of four splenectomized dogs. In dog III one reddened node was found in the abdominal and thoracic cavities. That in the former had a reddish medulla only but that in the thoracic cavity was reddish brown throughout. All external lymph glands were normal. In dog IV the latter was also the case but the abdominal lymphatic glands which were of normal volume had a very red medulla while the thoracic glands near the termination of the thoracic duct were large and red throughout. Strangely enough from the findings in two out of four dogs Tizzoni (40) concluded as follows:

Les alterations que nous avons constatées dans les glandes lymphatiques internes abdominales et thoraciques consistaient en un grossissement de ces glandes et dans une coloration rouge intense de leur substance médullaire. Ces alterations étaient toujours plus prononcées dans les glandes du côté gauche que dans celles du côté droit, et d'autant plus marquées, que le temps écoulé depuis l'opération était plus court; elles étaient toujours plus accentuées dans les glandes situées près de l'embouchure du canal thoracique. A l'examen microscopique ces lésions étaient représentées par une forte dilation des sinus et la substance médullaire à la suite de l'accumulation de globules rouges libres, de cellules globulifères et pigmentifères, abstraction faite des cellules lym-

phatiques normales; dans les cas les plus anciens on voyait encore des cellules globulifères et pigmentifères entre les colonnes médullaires, et en outre on remarquait une prolifération du tissu conjonctif qui entoure les vaisseaux. Ces glandes lymphatiques présentaient la réaction bleue du Fe. libre plus constamment et d'une façon plus intense que les glandes normales. Nous croyons devoir attribuer la tuméfaction de ces glandes lymphatiques et les autres altérations qui l'accompagnaient à des phénomènes irritatifs simples, à une lympho-adénite consécutive à la lésion traumatique, selon l'opinion de Legros. Les glandes lymphatiques externes et la glande thyroïde n'ont jamais présenté de lésions attribuables à la splénotomie.

Foa (8) was the first to question the validity of Tizzoni's (35, 36) conclusions regarding the new formation of accessory spleens after splenectomy. Tizzoni it will be recalled excised the spleen of four dogs and found 60 to 80 accessory spleens in the great and gastro-hepatic omenta in 2, 54 and 90 days respectively after operation. Although no examination of these animals had been made before operation, Tizzoni for wholly insufficient and incorrect reasons, nevertheless concluded that these spleens had formed *de novo*. Foa who had also called attention to the fact that such small nodules as described by Tizzoni are also found in dogs with entirely *normal* spleens apparently did not publish anything further on the subject, but Tizzoni published the results of forty necropsies a few weeks after the formal presentation of Foa's suggestions. In this paper Tizzoni (37) laid special emphasis on the fact that these necropsies were done at the veterinary school of Bologna in the presence and with the aid of his excellent friend Gotti.<sup>2</sup> The astonishing thing is that although 262 accessory spleens were found in *four* out of the *forty* dogs examined, Tizzoni nevertheless says that all his previous conclusions are confirmed! Tizzoni believed that in these four dogs nature herself had done a partial splenectomy by the development of a chronic interstitial splenitis. Moreover, he performed a splenectomy on a dog that had accessory spleens and although this animal died of an infection before the "usual results could manifest themselves" he nevertheless concluded that the results obtained in this animal also were sufficient wholly to

<sup>2</sup> The inference is clear, of course, and I mention these details simply because Tizzoni's experiments have been accepted for decades.

warrant his former conclusions! Tizzoni also failed to specify what the results in this case were and then stated that the accessory spleens which form as a result of splenectomy always arise as Malpighian corpuscles which become surrounded by pulp later while on the contrary, in the case of spleens that arise as a result of pathological conditions, the pulp forms first and the rest of the spleen from it.

In May, 1882, Griffini (15) reported a series of experiments on the regeneration of small experimentally-produced defects of the spleen in fourteen dogs and added that in a few cases he noticed a new formation of spleens from the great omentum or from the spleen itself *because of conditions not yet determined*. However, Griffini also failed to give any further details regarding these accessory spleens.

A year later Tizzoni (39) reported that he did splenectomies on two dogs which possessed accessory spleens before operation "as a result of disease." In one animal which was killed six months after operation there was no increase in size and number of the accessory spleens previously present in the great and gastro-splenic omenta but many newly formed spleens were found in the gastro-hepatic omentum, in the lateral ligament of the bladder, in the plica Douglasii, in the ischio-rectal fossa, in the serosa of the stomach and in the central tendon of the diaphragm. In the second animal killed seven months after splenectomy, Tizzoni concluded that there was *perhaps* an increase in size and number of the accessory spleens previously present in the great and gastro-splenic omentum and that in addition other newly formed spleens in all stages of development were found as in the previous animal. Since the first animal had suffered from post-operative local peritonitis Tizzoni concluded that these inflammatory processes inhibited the formation of accessory spleens in the gastro-splenic and great omentum and lead to their formation elsewhere.

Even granting that Tizzoni was still unbiased and not on the defensive it is perfectly evident that all the additional accessory spleens which Tizzoni regarded as having formed *de novo* because of splenectomy may just as well have formed because of condi-

tions present before operation. Hence splenectomy may not have been the causal factor at all. The same objection holds regarding the alleged increase in number of the spleens.

A year later Tizzoni (38) published a series of conclusions regarding accessory spleens drawn from sixty autopsies but he gives no details. These conclusions repeat all the older conceptions regarding accessory spleens and emphasize the fact that the non-experimental new formation of accessory spleens always results from a chronic interstitial splenitis. Tizzoni also drew further distinctions between spleens formed experimentally and as a result of pathological conditions but, since no new facts are presented in this two-page summary and since many of Tizzoni's conclusions can easily be shown to be untenable they will not be discussed here.

During June, 1883, and in 1884 Griffini and Tizzoni (16) reported that *in some instances* they found newly formed nodules on the main spleen and in the great omentum in a series of ninety-seven partial excisions of the spleen. The pieces excised were small (4 to 15 by 5 to 20 mm.) and the dogs were killed from forty hours to eighty-nine days after operation. In this article which the authors call a résumé they give no details whatever nor do they rule out reddened lymph glands. This joint résumé was followed by another résumé by Tizzoni (40) in which he declared that he wanted to test especially what would happen if splenectomy were done on dogs in which accessory spleens had previously formed as a result of disease of the main spleen. These experiments were regarded necessary in spite of the fact that Tizzoni had at least on two previous occasions tested experimentally and reported definite—very definite—conclusions regarding this very matter. Tizzoni again gave no details regarding these dogs—how they were selected, how many and how large the accessory spleens, how these were measured and where located, and so forth, yet he reported that he found an increase in the number and volume of the nodules previously present in the gastro-splenic and great omenta of the two dogs on which splenectomy was done. The experiment was limited to two dogs “Vir la difficulté d'avoir des chiens qui se trouvent dans les con-

ditions requires." The same objection urged above applies, to be sure, to these two experiments for in these cases also, the conditions responsible for the formation of accessory spleens before operation may have been responsible for their continued formation after splenectomy unless one assumes as Tizzoni apparently did not that these processes are limited entirely to the main spleen for Tizzoni reported the existence of identical pathological processes—chronic interstitial splenitis—in accessory spleens. But it is much more likely that Foa was correct when he confirmed Luschka's conclusion that accessory spleens are also found in the presence of wholly normal spleens.

In a final article Tizzoni reported on twenty-nine dogs in only four of which he found accessory spleens and never more than three in any one animal. The interesting thing in this article is the finding of the same pathological processes in the accessory as in the main spleen and the existence of a zone of infiltration around some of the accessory spleens. Tizzoni again concluded that the findings confirm all his previous conclusions.

Although Mosler (26) is usually quoted as having concluded that a new formation of spleens occurs after splenectomy this is a mistake. Out of thirty splenectomies on dogs done by Mosler the great omentum was covered with dark red nodules in only one dog! These nodules which Mosler upon Roth's examination and diagnosis which was based on Virchow's 'aufgestellten Principien,' called teleangiectatic hemorrhagic lymphomata were noticed ten months after operation. They varied from a lentil to a bean in size and looked like the spleen. Even granting that these nodules were accessory spleens there is absolutely no evidence to show that they were formed after or because of splenectomy. Mosler did not examine these dogs—or any others—before operation and facts presented elsewhere make it highly probable that these nodules were present before operation. Moreover, Mosler himself emphasized the fact that although Roth found hyperplasia of the mesenteric and of the aggregate lymph nodes in this dog he never saw such things in any of the other twenty-nine.

The conclusions of Tizzoni are also contradicted by Vulpius who found no enlargement of the lymph nodes and no accessory spleens after splenectomy in rabbits and goats up to five months after operation. Vulpius also called attention to the fact that Tizzoni failed to produce accessory spleens in rabbits and that Pouchet showed that animals possessing no lymph nodes can survive splenectomy.

From what has been said it is clear that although none of these investigators possessed any reliable criterion for the differentiation of lymph from hemolymph nodes yet the validity of their experiments was absolutely dependent upon the possession of such a criterion. Neither did most of them keep controls. Vincent "In order to institute a fair comparison . . . studied the hemal lymphatic glands in a dozen normal dogs. The average number found was 4 or 5." However, in a footnote Vincent (43) says: "I state this average with some diffidence, as the variations were so great in different cases." But even if the use of controls and a reliable means of differentiation between lymph and hemal nodes could be dispensed with this cannot be said regarding reliable data on the occurrence of accessory spleens in apparently normal dogs. Elsewhere<sup>3</sup> it has been reported that a large percentage (10+) out of an extensive series of apparently normal dogs and cats possess accessory spleens or true hemal nodes. This being the case it becomes highly probable as Foa first suggested that the splenic nodules found by Tizzoni, Eternod and Griffini on the peritoneum and great omentum after splenectomy were present in these animals before the operation. Morandi and Sisto for other reasons also surmised that the newly-formed nodes reported by these investigators were hemolymph nodes normally present in dogs. Besides Tizzoni's investigations contradict his conclusions. Nevertheless Warthin believed that these and the nodules mentioned by Mosler and Winogradow were hemolymph nodes which were newly-formed from fat and not accessory spleens. It is interesting in this connection to note that Roth who made the pathological

<sup>3</sup>Meyer, The occurrence of supernumerary spleens in dogs and cats, with observations on corpora libera abdominales. *Anat. Rec.*, Philadelphia, 1914.



examination for Mosier especially emphasized the fact that "das sehr sparsam vorhandene Fettgewebe zeigte nirgends eine nähere Beziehung zur Entwicklung derselben." Moreover Winogradow (53) only reported enlargement and reddening of lymph nodes and not a new formation of anything. The above criticisms also apply, of course, to Warthin's conclusions regarding the production of hemolymph nodes by splenectomy for he too did not state that he took the precaution to carefully examine the abdominal cavity of the animals before the operation was done. Moreover, Warthin did not consider the occurrence and presence of accessory spleens in normal animals and emphasized the impossibility of distinguishing between lymph and hemolymph nodes.

But even granting that these considerations do not wholly invalidate the conclusions of investigators who reported positive results the negative results of Vulpius (45), Vincent (43), Piltz (28) and Gibson (14) together with those presently to be reported in addition to other considerations certainly would seem to do so.

Piltz who concluded that there are no hemal nodes in cats and dogs removed the spleen from four dogs but failed to find the formation of hemal nodes in from eight days to three months after operation. Gibson also reported that he found "No naked eye abnormal appearances with the exception of the absence of the spleen" in a dog from which the spleen had been removed five and a half months previously. Similar observations were also made by him on the body of another dog in which both spleen and thyroid were removed. In this animal Gibson stated that he thought that the mesenteric nodes were large as compared with the normal but "not decidedly redder than usual." Practically the same results were obtained by Gibson in another dog from which the spleen had been removed and which had been bled 0.5 and 8 per cent of its body weight respectively within an interval of six days. Regarding this animal Gibson stated that the abdominal lymph nodes were "if anything somewhat redder in the center than usual," and contained some nucleated erythrocytes. However, that the slight—if any—red-

dening thought to be present in the lymph nodes of this dog, was probably not the result of splenectomy is suggested by the fact that in another dog which was bled 2.6 per cent of its body weight three days previously the lymphatic glands were said to have been "perhaps a little larger than usual but were no redder." Nucleated erythrocytes were again seen in scrapings from the mesenteric glands and the author came to the conclusion that erythrocytes are normally found in lymph nodes.

Meek (24) on the other hand reported that "the experimental production of such (hemolymph) glands in the guinea-pig is easy of accomplishment." Meek obtained these results by the injection of toxic substances such as living pathogenic bacteria, diphtheria toxin, oxalic acid and distilled water. Although Meek defined a "hemolymph gland as one which possesses a sinus containing blood instead of lymph" it is clear that he did not recognize that hemolymph or better hernal glands, have no relation to the lymphatic system whatever for he continued: "It would appear that in pathological conditions in the human subject their occurrence in large numbers is almost universal." Furthermore, farther on Meek stated that since hemolymph glands are only transformed lymph glands and since all manner of transition forms exist between the two there is no justification for a separate category. Hence, it must be clear that what Meek produced so easily in guinea-pigs was not hemolymph nodes at all. Not organs *sui generis* as Drummond first claimed for hemolymph nodes but merely changes present, even if not to the same extent, in normal lymph glands. Such changes no one will deny for as everyone knows it is an old, old observation, indeed, that various cellular changes and the accumulation of blood cells in the sinuses of lymph nodes occur under many pathological and other conditions but that will fail to convert a lymph node into a hernal node. In his experiments on guinea-pigs Meek also failed to state the amount and the strength of the solutions used, the age and weight of the pigs and the interval between the injection and the production of hemolymph nodes.

Since all the evidence for the experimental production of hemolymph nodes and accessory spleens either *de novo* or from

lymph glands, seemed wholly conflicting, a series of experiments on dogs and guinea-pigs was undertaken in connection with studies on hemolymph nodes of domestic animals. Ten dogs taken from two litters with known dates of birth were placed under laboratory conditions as soon as weaned, kept in identical surroundings and fed upon the same food until approximately six months old. The spleen was then removed from eight, one animal from each litter being kept as a control. Before removing the spleen the omenta, mesentery and as far as possible also the whole abdominal cavity, were inspected in order to note the condition of the lymph nodes and the presence of accessory spleens. After operation the animals were again kept under identical conditions and killed 41 to 126 days later, one animal being killed every two weeks. The spleens were inspected for scars and weighed. Recovery in every case was rapid and healing per primam except in one dog in which several small cutaneous stitch-abscesses developed. There were no adhesions in any animal. All of them gained markedly in weight not only because they grew rapidly but because they became fleshy and some even fat. Table 1 gives the age, the post-operative period and the result. The gain in weight is not given because it could have no special significance since all were young and growing dogs.

As is evident from the table, the results on these dogs fully confirm those of Vincent and of many operators on man. Only a single lymph node with a reddened center and 3.5 mm. in size, was found in the gastro-splenic omentum of one dog at the time of operation. No hemal nodes or accessory spleens were seen before or after operation, and it is especially interesting that the above node became paler or at least was temporarily paler, at the time of necropsy fifty-three days after operation. Although it could not be experimentally determined before or at the time of operation, whether the pinkish node was lymphatic or hemal in character this precaution was not neglected at the time of necropsy. All questionable appearing nodes which suggested intermediate forms were injected, but not a single hemal node was found. Since all nodes except those on the head, dorsum

and at the extremities were examined it follows that there should not be any question for skepticism as to the validity of the results in these cases. Moreover, to date over seventy dogs and approximately one hundred cats taken at random, have been examined in order to obtain a basis for reliable judgment.

Only a few specimens of the lymph nodes taken from dogs from which the spleen had been removed, were examined micro-

TABLE 1  
*Splenectomies on dogs*

NO. OF DOG	SEX	AGE IN MONTHS AT TIME OF OPERATION	INTERVAL IN DAYS	DEATH BY	RESULT
1	Female	5 $\frac{3}{4}$	30	gas	No hemal nodes or accessory spleens; bronchial lymph nodes intensely red <sup>1</sup>
2	Female	5 $\frac{3}{4}$	41	bleeding	No hemal nodes or accessory spleens; lymph nodes normal in appearance
3	Female	5 $\frac{3}{4}$	53	gas	No hemal nodes or accessory spleens; bronchial lymph nodes intensely red
4	Male	5 $\frac{3}{4}$	77	gas	No hemal nodes or accessory spleens; all lymph nodes normal in appearance
5	Female	6	89	gas	Same as 2, 4
6	Female	6	98	gas	Same as 2, 4, 5
7	Female	6	112	gas	Same as 2, 4, 5, 6
8	Female	6	126	gas	Same as 2, 4, 5, 6, 7

<sup>1</sup> The necessity for injecting the lymph nodes precluded a satisfactory microscopical study, of course.

scopically. In these nothing unusual was recognized and this was also true as to gross appearances of the lymph nodes in all the animals operated on except two. Moreover, the lymph glands in the two controls and in other dogs of similar ages were in all respects similar in gross appearance. Vincent (43) too concluded that "In spleenless dogs there was no apparent change in the lymphatic glands of any part of the body, neither in the direction of hypertrophy nor increased redness. On microscopical examination the hemal lymphatic glands presented the features of those in the normal dog." In a footnote Vincent adds:

The research has at any rate taught me the enormous difficulty of judging of hypertrophy of a tissue which is distributed in different parts of the body. In the case of the "hemal lymphatic" tissue the difficulty is increased tenfold by the fact that not whole glands but only parts of glands are formed of it, and so counting affords little guidance. It was suggested to me that I should freeze the gland, then cut out and weigh the red portions. This seemed feasible, but nevertheless on attempt was found too difficult and laborious to be practicable.

It will be recalled that many of the older experimenters reported enlargement of the lymph nodes and several also enlargements of the thyroid after splenectomy. According to Vulpian who found no enlargement up to five months in goats and rabbits, Tiedemann, Bardeleben, Gmelin, Mayer, Hyrtl, Domrich, Fisher, Ludwig, Eberhard and Gerlach noticed such an enlargement in dogs and Hegar and Simon in three cats. Zesas reported similar results for three rabbits, Winogradow<sup>4</sup> in three dogs, and Warthin in goats and sheep. Many of these investigators also reported changes in color and consistency as well as in cellular content and in many instances the enlargement was observed in all the peripheral nodes as well. However, since most of these observers did their experiments in the early days of asepsis Schiff's suggestion that the changes noted in many cases by the early experimenters were due to infection is undoubtedly correct. This suggestion is also supported by the presence of enlargement in *all* the lymph nodes, by the enlargement of the thyroid, by the large percentage of deaths and by the description of the microscopic appearance of the nodes themselves by many of the earlier experimenters. However, Freytag (11) concluded that in the rabbit which possesses no hemolymph nodes, splenectomy is followed by reddening of the lymph nodes near the greater curvature of the stomach and at the ileo-coecal junction in sixteen weeks. Freytag also surmised that this reddening is probably preceded by a swelling of the nodes since the superficial lymph nodes show this reaction and adds that red lymph nodes which are developed from 'preformed Anlagen' can be produced (?) decidedly earlier—within four weeks—by irritation from bleeding.

<sup>4</sup> Winogradow's percentages are wholly unconvincing for reasons obvious to anyone who examines his article.

Freytag further reported that lymph nodes respond as early as five weeks after splenectomy, that after bleeding in the rabbit small (0.75 cm.) "preformed splenic Anlagen" are found near the hilus of the main spleen, and that if these are removed at the time of operation negative results are obtained. He also stated in contradiction to the above investigators that red lymph nodes develop from the *mesentery* sixteen to nineteen weeks after splenectomy in a place one-half centimeter from the place where the spleen was excised, and that such can be produced in four weeks by bleeding. Still farther on it is stated that glands develop from *fat* and Freytag thinks hemal nodes may be transformed into spleens easier than lymph nodes.

It was stated above that although hemolymph nodes were not formed in any of these dogs in two instances nodes of unusual appearances were nevertheless found. In dog I, for example, a female killed by illuminating gas, thirty days after operation, the pubo-inguinal, abdominal, axillary and cervical lymph nodes were normal, but two large ( $1 \times 0.75 \times 0.5$  cms.) bronchial nodes lying dorsal to the root of the left lung were intensely red and all the visible afferent and efferent vessels engorged with blood. The many—about a dozen—fine, straight, afferent trunks which were only 0.25 to 0.75 mm. in caliber and about 1.5 cm. long were plainly visible up to the lung which they entered. Two larger (1.5 mm.) efferent trunks which were likewise engorged with blood, left these two nodes and joined the larger mediastinal vessels which empty into the main lymphatic vessels near the jugulo-subclavian junction. There also were two reddish nodes similarly placed on the right side. These had similar connections but the efferent trunk of one joined a more centrally placed mediastinal node near the apex of the thorax before joining the larger collecting trunks. The other node which lay nearer the root of the lung was large ( $1 \times 0.5$  cm.), flat, oval and joined by twelve to fifteen extremely fine blood-filled lymphatics which also seemed to have a sub-pleural origin.

In the other dog—No. III, also a female—killed fifty-three days after operation the pubo-inguinal, abdominal, axillary and cervical nodes were normal in appearance but the bronchial nodes

which were all as intensely red as the specimens in dog I, also had similar connections. Here then would seem to be two instances in which hemolymph nodes were produced experimentally. Not in the abdominal cavity, however, but in the thorax instead. But since very reddish nodes found in apparently normal animals, had always been found in connection with the lymphatic system the injection method was resorted to in these two cases also. By means of puncture of the subpleural tissues on the left side some of the fine straight engorged vessels joining the hemorrhagic nodes in dog I were injected. The india ink could plainly be seen displacing the blood in the fine afferent vessels and entering the node. Some of the vessels that were not injected in this way were however injected in a retrograde manner by puncture of the node which they joined. By this means the engorged efferent vessels were also injected and the india ink followed to its entrance into the jugular vein. The node on the right side was treated similarly with identical results and the same results were obtained in dog III. All the hemorrhagic nodes and vessels were thus shown to be in direct communication with the large collecting mediastinal lymphatic trunks. It is possible, to be sure, that the peculiar condition of the bronchial lymph nodes was due to splenectomy as some of the earlier investigators believed. Although improbable this is not denied, but that does not prove that they were true hemolymph (hemal) nodes which had been formed from lymph nodes or that they were newly formed nodes. As stated above, congested and hemorrhagic bronchial and mediastinal lymph nodes are very commonly found in both dogs and cats. Besides no such nodes were present in dog II which occupies an intermediate position in time, having been killed forty-one days after operation.

Haberer (18) in his study on supernumerary spleens also directs attention to the fact that many of the structures reported as accessory spleens probably are not such. He stated that he confused lymph nodes changed by sepsis, with spleens *macroscopically* although they were found to be typical lymph glands *microscopically*. Furthermore, Haberer says that in emaciated individuals he often found specimens which did not look like

spleens microscopically because they contained no Malpighian corpuscles, nor like lymph nodes but like transition forms resulting from pathological conditions.

Since the primary object of these experiments was not to determine what organs or tissues, if any, act vicariously for the spleen but to test the alleged formation of accessory spleens and hemolymph nodes after splenectomy, no special attention could be paid to microscopical changes which may occur in lymph nodes after splenectomy. The necessity for injecting them precluded such an examination but it may be recalled that Vincent found no changes in the lymph nodes after splenectomy. However, since his examinations were made on dogs five to six months after operation early changes as reported by Freytag may nevertheless have been present. Moreover, since Warthin reported the formation of hemolymph nodes in the abdominal cavity as early as a month after splenectomy, this observation and the above findings may undoubtedly partly be accounted for by such early changes in lymph nodes. This supposition also receives some support from observations after splenectomy by Winogradow (53), Zesas, Hegar and Simon and others.

In considering the factors responsible for the reddening of lymph nodes and the presence of blood in the sinuses Vincent (44) said: "I have not so far studied the blood-supply to these glands by means of injection, nor have I succeeded in finding the bloodvessels in direct communication with the lymph sinuses of the gland, but from the large number of red corpuscles sometimes found in them, I am convinced that there must be such communication."<sup>5</sup> It would be foolish to deny the possibility of the exceptional existence of such intra-nodal communications but from a large series of injections made into such nodes one is prompted to recall that Hyrtl tells us that someone was convinced that the seat of the soul was in the pineal gland but reflectively adds, "He found it not."

Winogradow, who found that the lymph nodes were enlarged soft, juicy, and dark and light red—especially red in the cortex—

<sup>5</sup> Lewis (21) stated that his observations regarding the existence of such communications are confirmed by himself, by Winogradow and also by Warthin.



so as to remind one of the spleen, because of the accumulation of erythrocytes which lay free in the reticulum among the lymphocytes, believed that this was the result of diapedesis after splenectomy. Tizzoni, however, as quoted above, regarded the changes in lymph nodes due to a lympho-adenitis resulting from a simple traumatic irritation.

In view of Meek's observations and conclusions a series of experiments were also undertaken on five guinea-pigs of known weight. One was given repeated injections of distilled water, the other of oxalic acid and three of diphtheria toxin. The injections were made directly into the peritoneal cavity. The amounts injected and the strengths as well as all other necessary data are given in table 2.

From the table it will be seen that hemal nodes were not found in a single instance. In case of no. 5 which died one week after the injection of 5/7 T the peritoneal cavity contained 1 to 2 cc. of bloody exudate but no hemal nodes had formed. The lymph nodes were difficult to recognize and only a single large lymph node at the root of the mesentery was slightly reddened. On injecting this node the usual results were obtained. The same statement holds for guinea-pig 4 which succumbed one day after the third injection of 1 cc. of a 1 per cent oxalic acid solution, and also for all the other animals.

Since as Retterer (29) showed, it is not uncommon to find more or less reddish lymph nodes in entirely normal guinea-pigs of all ages, under various conditions, and from many injections made on such, I am compelled to conclude that the above experiments did not result in the production of hemal nodes nor did they produce any unusual color changes in the lymph nodes. Hence from a review of the literature and from the experimental evidence available the conclusion that up to the present, no one has offered sufficiently conclusive evidence for the experimental production of true hemal nodes—not hemorrhagic lymph nodes—and accessory spleens is unavoidable. However, since true hemal nodes would *seem* to appear and disappear for some unknown reason the *possibility* of their experimental production—and that of supernumerary spleens?—is not denied. Changes in lymph

TABLE 2

NO. OF ANIMAL	SEX	WEIGHT JUNE 8 grams	INJECTION JUNE 8	WEIGHT JUNE 15 grams	INJECTION	WEIGHT JUNE 20 grams	WEIGHT JUNE 27 grams	WEIGHT JULY 10 grams	INJECTION JULY 10	WEIGHT JULY 18 grams	INJECTION JULY 19	REMARKS	RESULTS
1	M	200	0	235	0	270	295	325	0.5 cc. nor. salt cont. 5/14 T.	372	1 cc. nor. salt cont. 5/7 T.	Well Aug. 10; killed	Results as 4
2	F	230	0	260	0	285	280	220	0	297	1 cc. nor. salt cont. 5/7 T.	Sick July 20; died July 22	Results as 4
3	M	250	2 cc. aq. dest.	280	2.5 cc. aq. dest.	295	265	330	1.5 cc. aq. dest.	428	3 cc. aq. dest.	Well Aug. 10; killed	Results as 4
4	F	250	1 cc. 1/10 per cent oxalic acid	280	1 cc. 1/15 per cent oxalic acid	320	345	390	1 cc. 1 per cent ox- alic acid	Died July 11; no hemal nodes or acces- sory spleens; lymph nodes apparently normal			Results as 4
5	M	270	1 cc. nor. salt cont. 5/7 T.	295	1 cc. nor. salt cont. 5/7 T.	250			Died June 20; lost 45 grams; no hemal nodes present; lymph nodes normal to gross appearance; injected				

nodes, following various influences, have, of course, long been recognized but the nodes remain what they are—lymph nodes—and are not thereby converted into something else—hemal nodes having no connection with the lymphatic system.

It can be urged against these experiments on guinea-pigs that the injections were not continued over a sufficiently long period and that hemal nodes may have failed to form for this reason. Fortunately through the courtesy of my colleague, Professor Zinsser, I was able to examine the carcass of a goat which had been immunized against typhoid nine months before death and which had also been bled repeatedly. Yet only several small hemal nodes were found. Since all other goats examined during the past years contained more hemal nodes than this animal which had received repeated injections of toxin-containing material and had also suffered repeated losses of blood, it is evident that neither of these things resulted in the production of hemal nodes. Through similar courtesy I was also enabled to examine the carcasses of two sheep, one of which, although only about three years old, had been bled from 5 to 125 cc. approximately a hundred times during a period of two years. The other had been bled forty times during a year. In spite of this repeated loss of blood these animals were very fat indeed. Yet upon examination all lymph nodes were found to be exceptionally pale and small and only about a score of small apparently hemal nodes<sup>6</sup> from 1.5 to 2.5 mm. in size were found in the fat about the superior mesenteric vein in the first sheep and six in the second. In the first sheep, none were present in the very large amount of fat in the lumbar prevertebral region where they are usually so numerous, and only five (2-3 mm.) in the second. From these things it is evident, of course, that the injection of toxin or the loss of blood, even if repeated and extending over long periods, do not cause the formation of hemal nodes or necessarily produce any marked permanent macroscopic changes in lymph nodes. That these things may evoke a transient reaction on part of the lymph nodes is probable, to be sure, but that does not justify

<sup>6</sup> These were too small for puncture injections.

the conclusions drawn by many observers regarding the formation of hemal nodes. That changes may be produced in lymph nodes by repeated bleeding is also suggested by the studies of Smith indicating a lowering of the resistance of erythrocytes after the withdrawal of blood, but it is more than likely that the amount and frequency of that loss are very important factors in the determination of the character and extent of the changes in lymph nodes, a conclusion which also seems to be amply justified by Freytag's observations.

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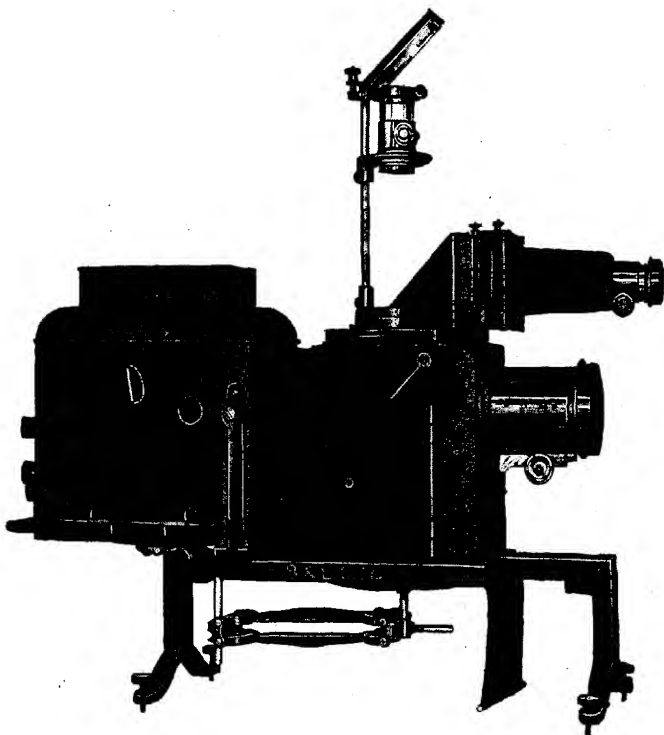
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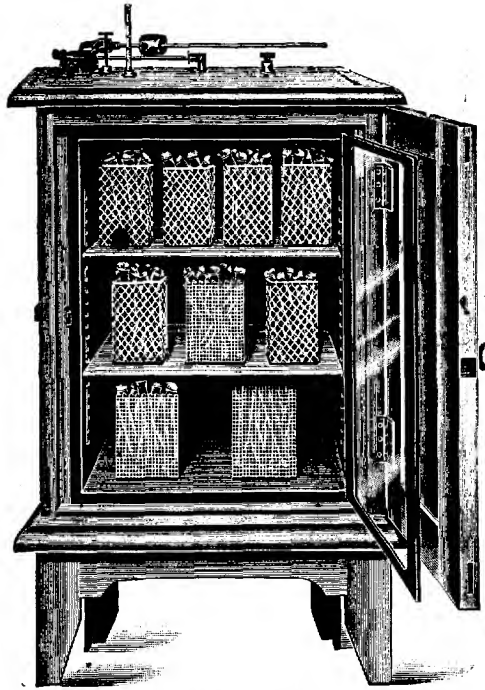
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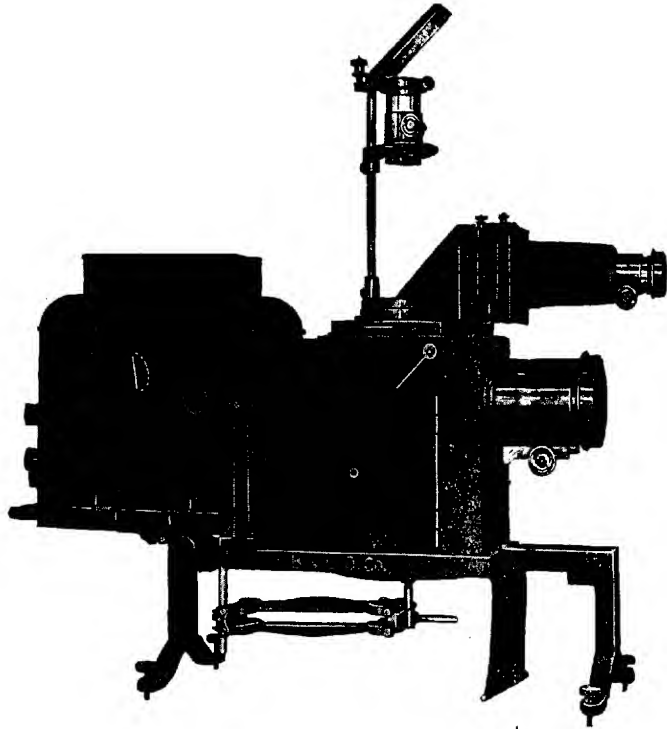
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# THE OLFACTORY SENSE OF THE HONEY BEE<sup>1</sup>

N. E. McINDOO

*Bureau of Entomology, Washington, D. C.*

TWENTY-FOUR FIGURES

## CONTENTS

Introduction.....	266
Material and methods.....	267
Experiments on bees in an observation hive.....	269
Experiments on normal bees in observation cases.....	271
Workers.....	272
Queens.....	274
Drones.....	275
Effects of clipping wings.....	276
Effects of light on longevity.....	277
Longevity of isolated bees.....	278
Longevity of bees with stings extracted.....	278
Effects of weather.....	279
Causes of mortality in observation cases.....	279
Olfactory experiments.....	280
Workers.....	282
Queens.....	285
Drones.....	286
Summary.....	288
Experiments on mutilated bees in observation cases.....	289
Antennae.....	289
Maxillae and labial palpi.....	297
Proboscis.....	297
Mandibles.....	298
Buccal cavity.....	298
Morphology of olfactory pores.....	299
Disposition.....	299
Variation.....	311
Wings of workers.....	311
Legs of workers.....	311
Stings of workers.....	313
Wings of queens.....	313
Legs of queens.....	313
Stings of queens.....	314
Wings of drones.....	314
Legs of drones.....	315
Summary.....	316

<sup>1</sup> The second part of this paper, which is a complete discussion of the literature pertaining to the sense of smell in insects, must be omitted on account of its length.



Structure.....	318
External structure.....	318
Internal structure.....	320
Summary.....	332
Physiology of olfactory pores.....	333
Wings cut off.....	333
Wings pulled off.....	334
Bases of wings glued.....	335
Pores on legs covered.....	336
Wings pulled off and pores on legs covered.....	337
Stings extracted.....	339
Summary.....	339
General summary and discussion.....	341
Literature cited.....	345

#### INTRODUCTION

Most students of bee behavior have considered this the fundamental factor in bee culture and have neglected the mechanisms through which behavior is manifest. Behavior consists only of the reactions or responses brought about through the senses and by means of internal stimuli. It is well to emphasize the necessity of studying the structure and function of sense organs and to form definite opinions concerning behavior only after the morphology of these organs is thoroughly understood. Behavior and the structure of the sense organs are inseparable and should be studied at the same time.

In the investigation here recorded two objects have been kept in view: (1) To determine the relative sensitiveness of the honey bee to different odors, so that it may be expressed numerically for comparison under different conditions; and (2) to locate the olfactory organs. The study of the behavior of normal bees under experimental conditions is used as a basis for correctly interpreting the observations on bees made abnormal for the purpose of obtaining data which concern the two objects in view. Since smell is probably the most important sense inside the hive and is perhaps only of secondary importance outside, we should know all that is possible concerning it.

Some writers assert that bees have an acute sense of smell, while others regard this sense in the bee as not important. Every bee keeper believes that this sense plays an important part in

the lives of his bees, but as yet he is able to profit by such a belief in only a few ways. For example, by the use of smoke he is able to control these insects while working with them. Sometimes a bee keeper uses a strong-smelling chemical while uniting colonies or in other manipulations.

Many entomologists have had something to say about the seat of the organs of smell in insects, but most of the views are purely speculative. A few have done extensive and thorough experimental work to determine the location of this sense. However, since they have failed to study sufficiently the behavior of the insects investigated, the responses observed have misled them in determining the seat of the olfactory organs. It is now generally believed that the antennae bear the organs of smell, but as all the antennal organs are covered with a hard membrane the objection has been raised that such organs can not receive olfactory stimuli. Hicks ('57) discovered some peculiar organs on the bases of the wings and on the legs of insects and suggested that these organs have an olfactory function. They have been neglected since 1860, when Hicks presented his third and last paper on them. About the same time several investigators began to work seriously on the antennal sense organs. This branch of work was later carried on by others, but the olfactory organs of Hicks have seemingly been forgotten.

#### MATERIAL AND METHODS

The Italian honey bee, mixed more or less with the black or German race, has been used in all the following experiments and observations. For the purposes of this paper workers are divided into four groups according to age; (1) One-day-old bees. They are gray, relatively inactive, and do none of the work of the hive but eat freely; (2) Young bees, between one and four days old. They are active, have begun to do certain work in the hive, and are still rather gray; (3) Middle-aged bees, from the age of four days to that at which they become black and shiny. This period includes the greater part of the bee's life. They have lost their gray appearance and the thorax is still quite hairy, having the same general color as other parts of the body; (4) Old bees

having the top of the thorax black and shiny, due to the loss of hair.\* Many times their wings are ragged at the tips and posterior edges.

To obtain material for the study of the disposition of the organs described by Hicks ('57-'60), workers and drones just emerged from the cells were used. Since queens of this age were not available when needed, older ones were employed. The legs and wings were pulled off at their articulations, and the thoraces and abdomens were slit open. These parts were put into a cold saturated solution of caustic potash, where they remained from one to three days, depending on the size and coloration of the material. When removed from this solution, the material was washed thoroughly in water and mounted between two cover glasses in a one-fifth saturated solution of potassium acetate. This solution gives a good refractive index and at the same time unites readily with the solution of asphaltum used for holding the two cover glasses together. It was thus possible to study the two sides of the specimens under the highest magnification.

To obtain material for the study of the internal anatomy of the organs herein discussed, worker pupae 13 to 21 days old (counting from the time the eggs were laid) were killed. The various appendages were removed and were then cut into small pieces, which were immediately dropped into fixing fluids. Various fixatives were used but the modified form of Carnoy's proved best. This fluid contains equal parts of absolute alcohol, chloroform, and glacial acetic acid, with the corrosive sublimate to excess. The material was left in this solution  $5\frac{1}{2}$  hours. It was dehydrated in the ordinary way but was cleared with cedar oil by using the sinking method. In order not to harden the chitin too much the material was left not more than two minutes each in 95 per cent and in absolute alcohol and not more than 10 minutes in cedar oil. It was then placed for 10 minutes each in ether and in thin celloidin, after which it was put into a vial containing thicker celloidin. The vial was put into melted paraffin and was watched closely, for the celloidin solution boils at a low temperature. It was boiled slowly for 10 minutes, until the celloidin became thick. The material was then removed from the vial, and a small mass

of the celloidin surrounding each piece was allowed to remain in the open air until the celloidin became firm. The pieces of material embedded in celloidin were then placed in a second vial containing a solution of three parts of chloroform and one part of 55° M. P. paraffin. It was again boiled for 10 minutes in the same manner as described above. It was then removed from this solution and infiltrated for 10 minutes in 55° M. P. paraffin inside the paraffin bath and finally was embedded in the same paraffin.

Serial sections were cut 6 and 10 microns thick and were mounted on slides in the usual way. These sections were stained on the slides with iron hematoxylin and eosin and also with safranin and gentian violet. The former double stain is the better for sense cell differentiation, but had it not been for the use of the latter double stain, the manner in which the sense fibers terminate would not have been clear.

When all the foregoing steps were carefully followed, good serial sections were had in abundance, but none were perfect, owing to the hard chitin. In fact these were only celloidin sections embedded in paraffin, although other celloidin sections, 35 microns thick, of entire adult workers were made by following the usual celloidin method. These were stained with Ehrlich's hematoxylin.

The drawings were made at the base of the microscope with the aid of a camera lucida, and all are original except figure 24, which was copied from Schenk ('03) to illustrate the anatomy of the antennal organs.

#### EXPERIMENTS ON BEES IN AN OBSERVATION HIVE

In order to ascertain the sensitiveness of workers to various kinds of odors in their natural environments, for comparison with the results in observation cases, an observation hive was constructed from a 10-frame Langstroth hive. The ends and sides were replaced by glass except a mere framework, the two end glasses being fastened permanently, while on each side were two pieces of glass so arranged as to slide. Thus an opening of any size could be made on the sides so as to expose the bees on the combs. The ends and sides had wooden shutters which fitted snugly against the glass, making the interior of the hive dark,

as in an ordinary hive, except when the shutters were removed so that the bees could be observed. This observation hive was mounted on a bench 3 feet high.

A little piece of cotton wet with oil of peppermint was placed on the alighting board among the workers. All of them except the fanners scattered in only a few seconds. The fanners, however, very seldom moved, but continued to vibrate their wings rapidly. Perhaps the current of air which each fanner created prevented the odor from coming in contact with its olfactory organs.

When this piece of cotton was held close in front of the bees, they always vibrated and stroked their antennae with the first pair of legs, turned around quickly, and moved away hurriedly. Also, whenever an open vial of peppermint was held near them, they always fled in a few seconds.

Three small pieces of cotton, one wet with oil of wintergreen, another with water, and the third with sugar sirup, were placed on the alighting board among many workers. The bees did not come closer than within  $1\frac{1}{2}$  inches of the first, they surrounded the second and sucked the water, while they literally covered the third piece of cotton to get at the sirup. When the cotton wet with sirup was placed against the one moistened with wintergreen, the bees sucked at the sirup only on the side farthest from the oil.

On three consecutive days in July three lots of eight dead workers each were placed on the alighting board to ascertain how long it would take the workers to remove them. The time varied from 35 seconds to 7 minutes and 45 seconds with an average of 3 minutes and 11 seconds for all 24 bees. A small drop of oil of peppermint was then placed on the tops of the thoraces of these same dead bees. Eight at a time were put on the alighting board as before. As a rule the workers never came nearer than  $1\frac{1}{2}$  inches of the scented bees and when they came this close they usually vibrated their antennae, turned to one side and went away. Occasionally a worker would rush up to one of the dead bees, seize it by a leg, wing, or antenna, loosen its hold as soon as possible, and jump backward. As usual, when a worker

seized one of these dead bees it had trouble in freeing itself because of the interlacing of their hairs. The mouthparts of the worker never came in contact with the oil of peppermint. After some time every scented bee was removed from the alighting board. The time taken to remove them varied from 10 seconds to 36 minutes, with an average of 10 minutes and 19 seconds for all 24 scented bees. Because of the repellent odor the bees were three times as long in removing the bees so treated as in the case of the untreated bees.

A small piece of cotton wet with oil of peppermint was held  $\frac{1}{2}$  inch from the bees that thickly covered one of the combs in the observation hive. The time which elapsed before the bees had scattered, leaving a circular space with a diameter of 3 inches, was recorded. This experiment was performed 20 times with intervals of 20 minutes between each two tests. The time varied from 10 to 20 seconds, with an average of 16 seconds. Similar experiments were performed with chemically pure oils of thyme, clove, wintergreen, and cedar. The results obtained are as follows: Oil of thyme 10 to 25 seconds, average 16 seconds; oil of clove 15 to 45 seconds, average 21 seconds; oil of wintergreen 15 to 30 seconds, average 22.5 seconds; oil of cedar 20 to 45 seconds, average 29.5 seconds. The general average for the 100 experiments with all five oils is 21 seconds. Judging from these results, peppermint and thyme have an equally repellent odor to bees. To bees, as well as to human beings, cedar oil has the least repellent odor of all five of these oils.

#### EXPERIMENTS ON NORMAL BEES IN OBSERVATION CASES

To determine the relative sensitiveness of queens, drones, and workers to various odors, under conditions which permitted of their close observation, triangular cases were used. These were made of three narrow wooden strips, two of which were 10 and the third 6 inches long, each strip being  $\frac{1}{2}$  inch thick. Cheese-cloth served as a bottom and glass as a top for the case. The apices and bases of these cases rested on two supports above a rigid table and the table legs rested on a concrete floor, near a

window. No screen was used to prevent the bees from seeing the observer because they never showed any response to movements other than rapid ones.

Nine middle-aged workers marked by clipping the wings in various ways were placed in each case. A lump of queen-cage candy (made by kneading powdered sugar in a small amount of honey) was kept continually in each case. At various times these bees were given water, sirup, or honey on a piece of cotton. To make conditions in these cases more natural for the bees, a small piece of comb was inserted.

Queens were introduced at various times and how and then one or more drones. Queens were tested while they were in observation cases with workers, but drones were tested when several of them were in the same case without workers.

#### WORKERS

To learn whether the results obtained in experimenting with bees in observation cases may be considered as normal, it was necessary to study the behavior of bees in these cases and to compare this behavior with that of bees in an observation hive. The behavior of individual bees in such captivity is in many respects similar to that of bees in the hive. With only nine in each case, it is possible to study the peculiarities in the behavior of each individual bee and each detail may be recorded, along with the bee's number. In these observation cases middle-aged workers live from 3 to 24 days, with an average of 9 days and 3 hours; queens 12 to 27 days, with an average of  $16\frac{1}{2}$  days; drones 6 hours to 13 days, with an average of 3 days and 9 hours.

When introduced into the observation cases workers are extremely restless and go about touching everything with their antennae. In a few minutes they usually begin to eat the candy, and after that they behave more normally. They are always more or less restless, and half of them are never perfectly quiet for even a few seconds, except when eating. Some bees eat during nearly the entire day, while others eat little at any time. The majority eat more frequently in the forenoon and are more restless in the afternoon.

They quite often clean and feed each other and stroke with their antennae the antennae and other parts of the body of the other bees in the case. They frequently crawl over the piece of comb and into the cells. Fanners and guards are often noticed; the former sometimes create air currents that can be easily detected, and the latter always stand at the edge of the group of bees ready to jump at any intruder.

When the nine bees introduced are from the same hive, no fighting is ever observed among them, unless one acts "crazy" by whirling around and around, in which event the others are apt to attack it. When a second lot of bees from the same colony, taken three or four days later, is introduced among an old lot, fighting is quite common. In case the second lot is introduced in less than three days there is little or no fighting. In all instances in which strange bees are introduced fighting occurs; the strange bee invariably tries to escape, but it never fights until firmly seized, when it fights by biting and trying to sting. In the majority of cases the stranger is stung first, although at times the combatants seem to sting each other at the same instant. In most cases the stranger loses its life, while the attacking bee escapes unhurt, except for bites on the legs and wings.

Forty fanners were removed from the alighting board of a hive and were put into an observation case somewhat larger than those described on page 271, into which candy, a piece of comb, and a piece of cotton soaked in water had been placed. Forty fanners from another hive were introduced into a similar case. Fanners are readily distinguished because they stand facing the entrance and fan with their wings. Nearly every one of the 80 was a middle-aged worker. When introduced into the cases most of these bees busied themselves in eating candy, a few began to fan, a few acted as guards, and the remainder wandered about aimlessly. On the next day several were seen eating, a few fanned, some acted as guards, but the majority ran about. Some of these carried bits of cotton and comb, having become cleaners.

The experiment was repeated with the same number of guards. These workers are also easily distinguished, for they guard the



entrance and jump at and usually seize any intruder. Half of them were old bees. When introduced into the cases most of them ate candy greedily, and the others ran about aimlessly. A day later most of them were quiet, some were seen eating, a few acted as guards, several carried bits of comb and cotton, a few pulled and tugged on the cotton, and the rest wandered about trying to get out. They never fanned except when excited. When either fanners or guards are removed from their hives and are put into observation cases, they cease to perform their special duties but carry on all the various vocations as far as they can under the conditions. This would indicate that in the hive a worker performing a special duty is capable of doing all kinds of work in case of need.

When one-day-old bees are introduced into observation cases they wander about considerably, although quite slowly. After a short time they become quiet and are inactive. They soon learn to eat candy and from this time on become more lively. They live from 17 hours to 13 days, with 5 days and 17 hours as an average.

When both young and old bees are put into an observation case, the young ones generally begin at once to clean the old ones. A young bee meets the old worker face to face, they stroke one another's antennae, and immediately the younger one begins to clean the older one's wings, legs, abdomen, and other parts. Ordinarily young workers clean bees older than themselves.

Very young bees, whether or not they are sisters of the bees already in the cases, are never attacked, and in fact the old bees "pay little attention" to the young ones.

#### QUEENS

When the nine bees in the observation case have a queen they appear to be slightly less restless and devote much of their time to her. They feed and clean her and usually at least a few are always around her. When a strange queen is put among them, they seem at first to be slightly afraid of her and keep their

distance. In a moment one or two begin to bite her legs and pull her wings, but this rough treatment never lasts long and no strange queen was ever seriously injured in these cases. After a short time the workers treat her as well as they would their own mother.

On many occasions the queens are seen eating candy, and they repeatedly examine the piece of comb. Very often the end of the abdomen is inserted into the cells of the comb, but no eggs were ever found. In most cases when a queen dies the workers no longer "pay any attention" to her, but in one instance after the queen had died she was surrounded for some time by the workers until one of them seized her wing and tried to drag her away.

#### DRONES

In every instance when one or two drones were put into one of the observation cases the workers attacked them. The drones run about inside the case aimlessly and continually try to get out. They "pay attention" to nothing and often run against one another and against the workers. A worker sometimes follows a drone, biting at his legs and wings. Very often a drone drags one or more workers that are holding tenaciously to one of his appendages around in the case several times. As soon as the workers can halt his wanderings, one of them climbs upon his back and tries to sting him. Perhaps more drones are actually worried to death by the workers continually following and nibbling at their wings and legs than die from the effects of the sting. On one occasion a worker was observed trying to sting a dead drone. A drone never offers to defend himself in any way but endeavors to escape.

When six to nine drones are introduced all at once among as many workers, the latter appeared to be overwhelmed by their number and seldom offer an attack unless one drone becomes separated from the others. Even in such cases a great deal of attacking is witnessed throughout a day, although no one drone is continually attacked until it dies. When nine drones are

placed into a case without any workers they live in peace among themselves.

Those which are continually tormented by the workers usually live only a few hours, while those that are not molested generally live several days. One lived 13 days and 2 hours, while the average for all was 3 days and 9 hours.

When only one or two drones are in a case they are never seen eating, but when several are in each case, and if they are not tormented by the workers, it is quite common to see them eating candy. Out of 99 drones observed only once was a worker seen feeding a drone and in only two cases did one drone feed another.

Of all three castes of bees in observation cases, the workers are the most active. Queens come second but their actions are extremely sluggish compared with those of the workers. Drones are the least active, although when irritated they move about more actively than queens. Drones usually remain more or less torpid, which causes them to appear sluggish.

#### EFFECTS OF CLIPPING WINGS

. Some additional cases of the same dimensions as the observation cases were made with wire-screen bottoms and dark red glass tops and provided with candy. Half of the right wing in 12 middle-aged workers was clipped off and the bees were put into one of these cases. Twelve bees from the same colony and of the same age which did not have their wings clipped were put into a second case with a lump of candy. These cases were kept in a dark table drawer and the death of each bee was recorded. This was repeated several times, and it was ascertained that the bees with clipped wings lived from 1 day and 19 hours to 12 days and 3 hours, with an average of 9 days and 4 hours; those not clipped, from 19 hours to 13 days and 19 hours, with an average of 9 days and 3 hours. Of the former 71 bees were used and of the latter 66.

In each set of 9 bees kept in the first type of observation case, various portions of one or both wings were removed from 8 of

the bees while those of the ninth bee were un mutilated. With all of these clipping showed no effect; this is true for both workers and drones. Of the former 403 were employed and of the latter 99.

To test this further and to ascertain if a greater number of bees in the same case makes any perceptible difference, 100 middle-aged workers were distributed equally in four large observation cases. Both wings of each bee were cut off close to the thorax. The bees always ate freely, seemed normal in every way, and lived from 18 hours to 27 days with an average of 10 days and 15 hours. Judging from these results, clipping the wings of workers and drones does not shorten their lives, and the increase in the number of bees in each observation case makes little if any perceptible difference. We may infer that the clipping of a queen's wings to keep her from going away with the swarm does not shorten her life, as some bee keepers have claimed.

Later many wings of all three classes were studied microscopically and it was found that the base of the wing contains a large nerve, many sense cells, and a tracheal branch, but no muscle extends beyond the base of the wing. When a wing is cut off close to the body, leaving only a short stump, the removal does not injure the structures enumerated above because the scissors pass beyond all of them. This subject will be discussed further in the section devoted to the distribution and structure of the olfactory pores, pages 299 to 333.

#### EFFECTS OF LIGHT ON LONGEVITY

In the experiments with the bees in observation cases and with those kept in the cases used for the experiments with bees having the wings clipped, no appreciable difference in the length of life is noticed. The bees in the observation cases were always kept in a bright light, while those in the other cases were always kept in a dark table drawer. The average length of life for the bees exposed to light was 9 days and 2 hours and for those kept in darkness 9 days and 3 hours. It may be inferred, therefore, that in captivity light does not shorten a bee's life.

## LONGEVITY OF ISOLATED BEES

The statement is often made that when a bee is isolated and can not communicate with other bees it dies within a short time. To test this, 40 middle-aged workers were carefully selected and were placed singly in small triangular observation cases, two of whose sides were 5 inches, and the third side 4 inches in length. The depth was  $\frac{1}{2}$  inch; the top was glass, and the bottom was wire screen. These bees always had plenty of candy and were given water occasionally, and the cases were placed side by side on a box by a window. In these circumstances the bees could not communicate with each other by sight or touch, but smell, and hearing—if they hear—were not excluded. These workers seemed normal in every respect and they lived from 4 days and 8 hours to 33 days and 22 hours with an average of 11 days and 20 hours.

The same experiment was performed with 100 active young bees that were just emerging from their cells and had never seen or associated with other bees. One might expect bees which had just emerged to live longer than middle-aged ones. However, this was not the case, for they lived from 1 day and 16 hours to 26 days and 4 hours, with an average of 4 days and 10 hours. The isolated middle-aged bees lived more than twice as long as the young workers.

## LONGEVITY OF BEES WITH STINGS EXTRACTED

The opinion is sometimes expressed that when a bee loses its sting it dies within a short time. To learn whether this is true the stings of 100 middle-aged bees were removed by holding a bee by a wing against the fleshy side of a meat rind. The bee stings the meat rind and loses its sting. In every instance the sting with all its accessory parts, including the poison glands, was pulled out. Several times half, and a few times the whole intestine was pulled out with the sting. These bees were introduced in equal numbers into two large observation cases, and were fed candy and given water as usual. Most of them behaved

normally in all respects by eating freely and by feeding and cleaning one another. Some of them fanned and sometimes one licked the protruding intestine of another bee. Some were very weak and showed signs of dying at once. The length of life after losing the stings varied from 1 hour to 6 days, with 30 hours as an average. Possibly in the hives a bee with its sting removed may live somewhat longer and may continue its work in a more or less normal way.

#### EFFECTS OF WEATHER

Climatic conditions perceptibly affect the activity of bees. When it is extremely warm, they are most active and are rarely quiet even for a few seconds. When it is moderately warm, they are less restless, and when rather cool, bees do not move freely. From 8 to 11 o'clock in the morning is the best time for studying the responses of bees to various odors, because they are then less active than during the middle of the day. From 4 o'clock until sundown is the second best period for studying their responses. During cool weather their movements are quite sluggish and when the humidity is high they are much less active and respond to various odors more slowly than when there is a low humidity. During damp weather they seem to live longer in observation cases than when the humidity is low. This may be because increased activity shortens its life.

#### CAUSES OF MORTALITY IN OBSERVATION CASES

In the observation cases bees apparently die from four causes, as explained below:

1. Old age is the most common cause of death. The greatest precautions were taken in selecting what were considered to be middle-aged bees, but even then many died in these cases within only a few days although they ate well and were no more active than usual.

2. Doubtless many of these bees live a shorter time on account of their extreme restlessness. Some of them were never quiet and did not even take time to eat, while others ate occasionally.

This extreme restlessness without sufficient food was certainly the cause of their shortened life.

3. Fighting is very fatal. It was usually prevented by using bees taken from the same hive at the same time. Bees which had been separated while fighting never attacked any other bee again, but lived thereafter in peace.

4. Several times, during damp days especially when sugar had accumulated on the floor of the case, all the bees in a case died within a short time. An examination showed that they had become coated with this wet sugar and were unable to clean themselves. This coating caused them to appear dark several hours before they died and evidently was the cause of their death.

#### OLFACTORY EXPERIMENTS

The following sources of odor were used for determining the reactions of the bees in observation cases: Chemically pure essential oils of peppermint, thyme, and wintergreen; bee foods, honey, and comb, and pollen from old combs; parts of plants, flowers of honeysuckle (*Lonicera* sp.), leaves and bits of the stem of pennyroyal (*Hedoma pulegioides*?), of spearmint (*Mentha spicata*), and of scarlet sage (*Salvia* sp.); and bee stings. These substances were kept in stoppered vials of the same shape and

The parts of plants were used only so long as they remained fresh. Bee stings were obtained by seizing workers by the legs with a pair of forceps and holding them to a meat ring. In a few seconds the bee invariably stung the ring and its sting was pulled out. The stings with the adhering poison sacs and muscles were immediately put into a tightly stoppered vial. One hundred such stings were used at a time and give characteristic irritating and pungent odor. At first this odor is similar to that emitted when a bee stings an object, but after a few hours it becomes somewhat different, not quite so irritating but more pungent. In 24 hours this odor becomes foul and unpleasant and after 48 hours it is one of the foulest and most offensive that one can experience. These stings were used not longer than 20 hours after removal.

To ascertain the true responses of bees to the odors of the preceding substances, the chosen insects were carefully observed each as long as they lived. A bee was tested only when it became perfectly quiet, without even the antennae being moved in the least, and when it was not watching another bee or any other object, and was not eating. The stopper was quickly removed and the vial was then gently and slowly placed under the observation case directly beneath and within  $\frac{1}{2}$  inch of the individual bee tested. Occasionally the vial was placed a few inches in front of the specimen, to test its ability of smelling for a short distance. Whenever all of these precautions are taken, a normal bee responds to any one of these odors without failure. As a control, an empty and odorless vial was now and then placed under the bee in the same manner. If by chance a bee moves while the control test is being made, its behavior is quite different from that observed when odors are used. Only the first responses have been recorded, and in all cases where there was the least doubt as to whether the bee moved for any reason other than the olfactory stimulus, such movements were never recorded. The reaction time was counted in seconds. With an ordinary watch the minimum time which can be definitely recorded is 2 seconds, although many individuals respond to some of the odors much more promptly. Owing to this source of error the average recorded time is probably double what it should be in the cases where the response was prompt. An intermission of 15 minutes elapsed between any two tests in the same observation case and generally several times this period elapsed before the same bee was again tested with the same or with a different odor. Some individuals were tested five times with the same odor, although most of them were not so tested more than twice. In all, 509 specimens have been carefully observed, but on account of the extreme restlessness of some of the bees it has been possible to obtain records for only 263.

In the following paragraphs are given the responses of the three castes of bees to the odors of the ten different substances and the average reaction times in seconds. In recording the responses the term "vibrate" is used to describe the rapid move-



ment of the antennae up and down or from side to side. When this movement is slow they are described simply as having "moved." When the expression "acts as if noticing odor" is used it is to be interpreted as meaning that the antennae are "vibrated" and that the bee seems to "fix its attention" on the spot from which the odor comes. Anthropomorphic terms are of course used only in a figurative sense, often for lack of words adequately to describe the actions otherwise. Sometimes a bee lies flat on its thorax and abdomen, the word "arose" is to be interpreted as meaning that the bee gets up and stands on its feet. In the averages of reaction times the probable error is presumably high. It has not been calculated, since slight differences in reaction time are not considered as significant in the discussion of results.

#### Workers

##### *Oil of peppermint:*

- 13 moved away quickly
- 6 moved away quickly and vibrated antennae
- 6 moved away slowly and stroked antennae with front legs
- 4 moved antennae slightly and moved away slowly
- 2 jumped quickly as if attacking an enemy
- 2 turned around quickly and moved antennae
- 1 put its head on floor of case and vibrated antennae
- 1 arose quickly and stroked antennae
- 1 arose slowly and stroked antennae
- 1 arose quickly

Reaction time 2 to 3 seconds, average 2.3 seconds

Workers tested, 37

At four different times while using this oil all the bees in the case became excited. They always produced an uproar by rapidly vibrating their wings.

##### *Oil of thyme:*

- 7 moved away quickly
- 6 moved antennae and then moved away slowly
- 4 moved away quickly and vibrated antennae
- 3 arose and moved away slowly
- 2 moved slightly and stroked antennae
- 2 arose quickly and vibrated antennae
- 1 jumped toward odor and stroked antennae
- 1 moved antennae quickly

Reaction time 2 to 3 seconds, average 2.4 seconds

Workers tested, 26

*Mercuric:*

- 11 moved antennae and moved away slowly
- 4 moved away quickly
- 3 moved away quickly and vibrated antennae
- 2 moved away slowly and stroked antennae
- 2 moved slightly and stroked antennae
- 2 moved away slowly and vibrated antennae
- 1 moved antennae slightly
- 1 turned around as if noticing odor

Reaction time 2 to 5 seconds, average 3.23 seconds

Workers tested, 20

Three times all bees in the case were excited

*Honey and comb:*

- 6 moved antennae and acted as if searching for the source of odor
- 6 stroked antennae several times
- 4 moved slightly as if noticing odor
- 4 vibrated antennae as if noticing odor
- 2 moved slightly and vibrated antennae
- 2 arose slightly and moved away slowly
- 2 vibrated antennae and put their heads on the floor above the vial
- 1 walked 2 inches toward odor, then turned around above the vial
- 1 arose slowly and acted as if searching for source of odor
- 1 raised antennae, turned around, and moved away slowly
- 1 turned around several times, stroked antennae, and acted as if searching for source of odor

- 1 arose quickly and held antennae on floor of case as if noticing odor

Reaction time 2 to 11 seconds, average 4.5 seconds

Workers tested, 31

*Pollen:*

- 10 vibrated antennae and acted as if noticing odor
- 3 turned around with their heads on the floor as if noticing odor
- 3 moved away slowly and stroked antennae
- 3 moved away slowly
- 3 stroked antennae and acted as if searching for source of odor
- 3 vibrated antennae and acted as if noticing odor
- 2 arose slightly and acted as if searching for source of odor
- 2 moved away quickly, then turned around as if noticing odor
- 2 arose and moved away slowly

Reaction time 2 to 16 seconds, average 5.37 seconds

Workers tested, 31

*Flowers of honeysuckle:*

- 6 vibrated antennae as if noticing odor
- 4 moved away quickly as if noticing odor
- 4 moved away quickly and vibrated antennae
- 3 turned around as if noticing odor
- 3 arose quickly and acted as if noticing odor
- 3 raised antennae and moved slightly
- 2 jumped slightly and acted as if noticing odor
- 2 stroked antennae and acted as if noticing odor
- 2 moved away slowly
- 1 moved to one side and acted as if searching for source of odor
- 1 vibrated antennae and walked an inch toward the vibrated antennae

Reaction time 2 to 10 seconds, average 3.6 seconds

Workers tested, 31

*Leaves and stems of pennyroyal:*

- 6 moved away quickly
- 4 moved away slowly
- 4 stroked antennae and moved away slowly
- 3 vibrated antennae as if noticing odor
- 3 arose quickly and moved away quickly
- 2 moved their heads near floor as if noticing odor and moved away slowly
- 1 arose quickly and stroked antennae
- 1 jumped slightly and moved away quickly
- 1 vibrated antennae and turned around as if noticing odor

Reaction time 2 to 5 seconds, average 2.6 seconds

Workers tested, 25

Twice this odor caused all the bees in the case

*Leaves and stems of spearmint:*

- 6 moved away quickly
- 4 moved away slowly
- 3 turned around as if noticing odor
- 3 jumped quickly toward odor
- 2 vibrated antennae and moved away slowly
- 1 moved away slowly as if noticing odor
- 1 moved away quickly and stroked antennae

Reaction time 2 to 4 seconds, average 2.48 seconds

Workers tested, 24

*Leaves and stems of scarlet sage:*

- 10 moved away slowly
- 4 moved slightly and acted as if noticing odor
- 3 moved away quickly as if noticing odor
- 2 vibrated antennae as if noticing odor
- 2 moved to one side slowly and vibrated antennae
- 2 arose slowly, stroked antennae, and moved slightly
- 1 arose quickly
- 1 jumped slightly and moved away slowly

Reaction time 2 to 5 seconds, average 3.14 seconds

Workers tested, 25

Once all the bees in a case excited

*Bee stimulated*

- 13 stroked antennae and acted as if noticing odor
- 6 moved an inch or two until they were directly over the vial
- 3 stroked antennae and acted as if noticing odor
- 2 moved slightly and vibrated antennae
- 1 moved away quickly
- 1 turned and quickly as if noticing odor
- 1 jumped quickly and vibrated antennae as if noticing odor
- 1 moved quickly, vibrated antennae, and followed odor when vial was moved beneath the case

Reaction time 2 to 3 seconds, average 2.16 seconds

Workers tested, 28

*Smoke:*

During all these experiments it was absolutely necessary to refrain from smoking tobacco because the least smoke in the laboratory invariably excited the bees. They produced an odor by rapidly vibrating their wings, became very restless, and remained in this excited state for some time after being irritated. The least amount of smoke from the keeper's smoker, whether inside or near the outside of the house, also usually excited them.

### Queens

*Oil of peppermint:*

- 3 moved away quickly
- 3 raised antennae and moved away slowly
- 2 moved away quickly and vibrated antennae
- 2 moved away slowly
- 1 vibrated antennae
- 1 arose and moved away

Reaction time 2 to 7 seconds, average 3.8 seconds

Queens tested, 12

Once all the workers in a case were excited

*Oil of thyme:*

- 3 stroked antennae and moved to one side
- 2 arose quickly and moved to one side slowly
- 2 moved away slowly and vibrated antennae
- 2 vibrated antennae
- 1 moved away slowly and vibrated antennae
- 1 lifted antennae and arose slowly

Reaction time 3 to 5 seconds, average 3.9 seconds

Queens tested, 11

*Oil of wintergreen:*

- 4 raised antennae and moved away slowly
- 2 moved away slowly and stroked antennae
- 2 moved away slowly and vibrated antennae
- 1 moved away slowly
- 1 vibrated antennae
- 1 arose and moved slightly

Reaction time 3 to 7 seconds, average 4.2 seconds

Queens tested, 11

*Honey and comb:*

- 4 vibrated antennae and moved away slowly
- 2 vibrated antennae
- 1 arose and moved slightly

Reaction time 5 to 10 seconds, average 6 seconds

Queens tested, 7

*Pollen:*

- 4 vibrated antennae and moved slightly
- 2 vibrated antennae
- 1 moved only slightly

Reaction time 4 to 10 seconds, average 6.3 seconds

Queens tested, 7

*Leaves and stems of pennyroyal:*

- 3 vibrated antennae
- 2 vibrated antennae and moved away slowly
- 1 vibrated antennae and turned around slowly
- 1 vibrated antennae and arose

Reaction time 3 to 8 seconds, average 5.1 seconds

Queens tested, 7

*Drones**Oil of peppermint:*

- 14 moved away quickly
- 3 raised antennae and moved away quickly
- 3 arose and moved away quickly
- 2 moved away slowly
- 2 moved away quickly and vibrated antennae
- 1 raised antennae quickly

Reaction time 2 to 3 seconds, average 2.3 seconds

Drones tested, 25

Only once were all the workers in a case excited.

*Oil of thyme:*

- 14 moved away quickly
- 4 moved away slowly
- 3 moved slightly and vibrated antennae
- 3 arose slowly and moved away quickly
- 1 raised antennae

Reaction time 2 to 4 seconds, average 2.16 seconds

Drones tested, 25

All workers in the case were excited only once

*Oil of wintergreen:*

12 moved away quickly

6 arose quickly and moved away slowly

3 arose, stroked antennae, and moved away slowly

2 raised antennae quickly and moved away slowly

2 moved antennae slightly

Reaction time 2 to 4 seconds, average 2.48 seconds

Drones tested, 25

*Honey and comb:*

5 moved antennae slightly

4 vibrated antennae vigorously

2 raised antennae and turned around as if noticing odor

2 vibrated antennae vigorously and tried to reach the honey through the floor of the case

2 vibrated antennae as if noticing odor

2 arose slowly and vibrated antennae

1 placed antennae on floor of case and acted as if noticing odor

1 stroked antennae and turned around as if searching for honey

Reaction time 2 to 10 seconds, average 3.8 seconds

Drones tested, 19

*Pollen:*

5 vibrated antennae and moved slightly

2 vibrated antennae vigorously

2 vibrated antennae and acted as if noticing odor

2 raised antennae and moved away slowly

2 arose quickly and stroked antennae

2 arose and moved away slowly

1 tried to get through the floor of the case

1 moved only slightly

Reaction time 2 to 6 seconds, average 3.56 seconds

Drones tested, 17

*Flowers of honeysuckle:*

5 moved away quickly

4 moved away slowly

3 vibrated and stroked antennae

3 vibrated antennae as if noticing odor

3 arose quickly and moved away slowly

1 raised antennae quickly and moved away quickly

1 arose quickly and turned around as if noticing odor

Reaction time 2 to 5 seconds, average 2.8 seconds

Drones tested, 20

*Leaves and stems of pennyroyal:*

- 8 moved away quickly
- 6 raised antennae and moved away slowly
- 3 moved away slowly
- 3 vibrated antennae vigorously
- 2 arose and moved away slowly
- 2 arose slowly
- 1 arose quickly and stroked antennae

Reaction time 2 to 5 seconds, average 2.74 seconds

Drones tested, 25

*Leaves and stems of spearmint:*

- 6 moved away quickly
- 5 vibrated antennae and moved slightly
- 4 arose and moved away slowly
- 3 moved away slowly
- 1 raised antennae and moved slightly

Reaction time 2 to 4 seconds, average 2.55 seconds

Drones tested, 19

Once all the workers in a case were excited

*Leaves and stems of scarlet sage:*

- 4 arose quickly and moved away slowly
- 4 vibrated antennae slightly
- 3 moved away slowly
- 3 raised antennae and moved away slowly
- 2 vibrated and stroked antennae and moved away slowly

Reaction time 2 to 10 seconds, average 3.37 seconds

Drones tested, 16

### *Summary*

To summarize the preceding data, it is found that 87 per cent of the individuals tested moved away from the peppermint, 80 per cent from thyme, 89 per cent from wintergreen, 6 per cent from honey, 29 per cent from pollen, 45 per cent from honeysuckle, 78 per cent from pennyroyal, 73 per cent from spearmint, 69 per cent from scarlet sage, and 4 per cent from the bee stings. Thus, to all of these odors, except those of honey, pollen, honeysuckle, and the bee stings, bees react negatively. For the odors of honey, pollen, and honeysuckle the most characteristic response is in searching for the source of the odor by turning around over the vial with the head almost on the floor of the case and by vibrating the antennae vigorously. In response to the bee-

showing that the bees usually moved quickly toward the source of the odor when the vial was moved 2 or 3 inches from them. By including the data from the use of all of these ten odors, except that of castings, the average reaction time for the very first response for all the workers is 3.29 seconds, whereas the average reaction time for all the drones is 2.86 seconds. By using the data for the first responses to peppermint, thyme, wintergreen, honey, pine, and pennyroyal, the average reaction time for all workers is 3.1 seconds, for all drones 2.9 seconds, and for all queens 4.9 seconds. Hence, in spite of the fact that drones are less active in the observation cases, they responded somewhat more quickly than did the workers and much more quickly than did the queens.

It is evident, therefore, from these data that the olfactory sense in the honey bee is acute and that the sensitiveness to various odors is most highly developed in the drones, and least highly developed in the queens.

#### EXPERIMENTS ON MUTILATED BEES IN OBSERVATION CASES

In the preceding pages the behavior of un mutilated bees in observation cases and their reactions to odors are discussed. The behavior of bees that have been injured in various ways will now be discussed, together with an account of the experiments with them with various odors. In these experiments all the bees in the same observation case were mutilated and they were given the same kinds of food as those on which the uninjured bees survived.

#### ANTENNAE

Entomologists now generally agree in the belief that the organs of smell in insects are located on the antennae. Bees with either the right or left antenna pulled off are much less pugnacious than are those with the antennae intact, and they "pay less attention" to each other. They appear otherwise normal, except that their ability to communicate is considerably decreased. They feed the queen and each other, eat normally, and often stroke each



other as do those with both antennae intact. While the bees with both antennae intact lived on an average of 9 days and 4 hours in observation cases, the bees with one antenna removed lived under the same conditions on an average only 6 days and 18 hours.

Bees with one antenna pulled off and with 2 to 8 joints of the other one cut off never "pay any attention" to each other and very seldom are seen fighting, but are just as apt to fight a hive-mate as a stranger. All of these eat more or less, but the more joints cut off the second antenna the less normal they appear. The greater the number of joints severed, the less number of days they live. Such bees, on an average, lived 5 days and 11 hours in the observation cases.

Bees with both antennae pulled off at first wander about inside the case more or less aimlessly. They seem to have no means of communicating with other bees. They run against each other and then try either to stroke or to fight one another, although they fail to do either. Only occasionally do they feed one another and only a few are seen eating. After a few hours they become inactive and usually remain so until they die. In their activity they are similar to young bees. They "pay little or no attention" to other bees and in most cases do not move unless another bee runs against them. The ones studied lived on an average of only 19 hours in the observation cases.

Bees with both antennae cut off at their bases behave very similarly to those just described. Those observed lived only 2 hours on an average in the observation cases.

When the antennae were varnished with shellac or celloidin the bees were not at all normal. They failed to eat; many ran around and acted "crazy" and tried to clean their antennae, but in this they failed. Those with shellac on their antennae lived only a few hours, while those with celloidin lived, on an average, 16 hours in the observation cases.

The antennae of some were covered with vascline. These bees at once cleaned their antennae and were soon normal.

The left antennae of 15 workers were pulled off at the base with a small pair of forceps. The responses of these bees to the

odors, the three essential oils are similar to those described for bees with both antennae intact. The average reaction times are as follows: Oil of peppermint 5.2 seconds, oil of thyme 4.3 seconds, oil of wintergreen 4.8 seconds. The average time for 15 specimens with their right antennae pulled off gave similar results, as follows: Oil of peppermint 4.8 seconds, oil of thyme 4.1 seconds, oil of wintergreen 4.6 seconds. The average time for workers with one antenna pulled off is: Oil of peppermint 5 seconds, oil of thyme 4.2 seconds, oil of wintergreen 4.7 seconds. This gives a general average of 4.6 seconds for the three oils, while the general average of the same oils for bees with both antennae intact is only half as much, that is, 2.3 seconds. According to these results, and on the theory that the antennae contain the organs of smell, it would at first glance seem that each antenna plays an equal part in receiving odor stimuli and that when either antenna is absent the average time for the responses is consequently doubled.

Bees with either the right or left antenna pulled off and with from 2 to 8 joints of the remaining flagellum cut off gave the following average reaction times when using the three essential oils:

	seconds
2 joints missing.....	15
4 joints missing.....	44
5 joints missing.....	56
6 joints missing.....	27
7 joints missing.....	98
8 joints missing.....	88

Thus, in general, the greater the number of joints of the second antenna removed the longer it takes the bees to respond to odor stimuli. Workers thus mutilated are not at all normal and live on an average only 5 days and 11 hours.

Bees with both antennae either pulled off or covered with celloidin entirely fail to respond to the essential oils. They also are quite abnormal in their behavior.

Two drones, one of which had 4 and the other 5 joints of one flagellum missing, were taken in this condition from a hive.

These were apparently normal in other respects, but with 6 of the 10 odors used in these experiments they gave an average reaction time of 3.16 seconds, whereas the average for the same odors with unmutated drones was 2.9 seconds.

Ninety middle-aged bees with both antennae pulled off were placed on the combs of the observation hive at 4 o'clock in the afternoon. To prevent them from flying from the hive, their right wings were clipped. These bees were more restless than normal bees. They wandered about and continually crawled out of the hive. Whenever one was found outside the hive it was put back, although all of them eventually escaped in this way, because two days later not one of them was either alive or dead, was found in the hive. The behavior of these mutilated bees was similar to that of the antenna-less bees in the observation cases. They took no part in the activities of the hive and appeared to have lost all means of communicating with the other bees. The next day at various hours 16 of them were found dead in front of the hive. The third day at 8 o'clock 3 more were found, and on the fourth day at 4 o'clock one more. In three days 20 were found dead, while the other 70 certainly crawled out of the hive and escaped. Counting the time until their dead bodies were found, these 20 lived on an average only 21 hours. In the observation cases the average length of life of such bees was 19 hours. From these combined results it is evident that bees with their antennae pulled off are not normal and therefore whatever results are obtained by experimenting with them must always be discounted.

Immediately after pulling off both antennae of a worker, it was placed on a comb with the other bees in the observation hive. A small piece of cotton wet with oil of peppermint was held  $\frac{1}{2}$  inch in front of this antenna-less bee, and afterward smoke from a bee keeper's smoker was gently blown on it. In performing these experiments with 10 different workers, not a single mutilated bee reacted in the least, whereas all the other bees soon fled from the oil of peppermint and caused an uproar by rapidly vibrating their wings when smoke was gently blown on them. Similar experiments were performed, by using oil of thyme, clove, wintergreen,

and the same bees were afterwards tested with smoke. Not one of 50 mutilated bees so treated showed any reaction, while normal bees never failed to react.

The mutilated bees in these experiments were restless and wandered about considerably. They often crawled into cells. Occasionally one cleaned itself and sometimes other workers cleaned them. Very often a mutilated worker fed one to three other bees before it crawled off the comb and disappeared. How long these mutilated bees lived can not be stated because they were soon lost among the other bees. Three or four hours afterward two or three of them were found lifeless in front of the hive.

Miss Fielde (1902) believes that the olfactory organs which an ant uses in recognizing enemies lie in the fifth and sixth antennal segments. To ascertain if one or more particular antennal joints of bees bear olfactory organs which are used in recognizing strange bees, 3 to 8 joints were cut from both antennae of 3 lots of 9 middle-aged bees each. These mutilated workers were introduced into an observation case and their behavior was studied. No abnormality in behavior was noticed among them except that they were slightly less active. Occasionally when first introduced one attacked another, although not seriously, and no injury was ever done to any of them. They lived scarcely 9 days on an average.

Nine bees with 2 to 8 joints of both antennae amputated were introduced into an observation case. The following day several strange bees, one at a time, were put into the same case. Most of the mutilated bees at once noticed a stranger and in a few seconds one of the strange bees was attacked. Both fighting bees were immediately removed from the case, the stranger was discarded, while the mutilated bee was killed and its remaining antennal joints counted. This experiment was repeated seven times. In all, 72 mutilated bees and as many uninjured ones were used. In only a few instances did the mutilated bees attack each other. This can always be prevented by keeping the bees isolated for a short time after the operation. When strange bees were put among the mutilated ones almost one-half of the

former were attacked. When the remaining antennal joints were counted it was found that in the pugnacious bees from 2 to 6 antennal joints were missing and in those that did not attack the strangers from 2 to 8 joints were absent. Only the last 8 joints of the antennae of a worker contain the supposed olfactory organs and when these 8 joints were removed all of these organs are eliminated. According to Miss Fielde's theory the fifth and sixth antennal segments of the worker bee would be the ones which carry the olfactory organs by which strange bees are recognized. Furthermore, Miss Fielde claims that the tenth antennal joint of an ant contains the organs through which the colony odor is recognized and if this joint is removed colony-mates will attack each other. Judging from the foregoing experiments, no particular antennal joint of a worker bee contains the organs by which the odor of sister bees is received, because the mutilated bees never fought each other regardless of the number of antennal segments amputated, when they were kept out of the cases for a few minutes after the antennae were injured.

To determine whether bees with mutilated antennae are normal, 11 joints of both antennae of 12 middle-aged bees were cut off. When introduced into an observation case these bees did not fight, "paid no attention" to each other, were quiet, and only one ate candy. When strange bees were put among them the mutilated bees always gave the strangers the right-of-way and did not attack them. They lived only 6 hours on an average.

Both antennae of 95 middle-aged bees were burnt off with a red-hot needle. These workers were also abnormal and lived only 17 hours on an average. The antennae of 30 drones were similarly burnt off and they too lived only 17 hours on an average.

Thirty middle-aged bees were immersed in water for 15 minutes. When removed they appeared entirely lifeless and their antennae were pulled off at once. They revived and lived only 19 hours on an average.

Since bees whose antennae are mutilated after they become adults are abnormal, the following experiments were performed with immature bees. A frame of brood was removed from a hive, the cells of sealed brood were gently uncapped and both

antennae of 100 pupae ranging in age from 14 to 18 days from the laying of the egg were carefully cut off near the head. Great care was exercised not to injure the immature bee in any way other than by the amputation of these appendages. The cells were again closed with a very thin layer of beeswax, a wire-cage screen was placed over these uncapped cells so that the adult workers could not pull the mutilated bees out of their cells, and the frame was put into the hive. In a few days these bees began to emerge from their cells. They were removed from the hive and placed in observation cases with intact middle-aged bees. All bees thus mutilated were abnormal and lived as adults about 5 days on an average.

In like manner both antennae were cut from 300 pupae 13 and 14 days old from the laying of the egg. In this instance a wire screen was placed over the entire frame so that the mutilated bees could not escape when they emerged. Seven days later they began to emerge and most of them in a short time crawled from their cells. They soon mixed on this comb with other young bees that were un mutilated and they had plenty of food but they were all dead 5 days later.

Seven of the workers with their antennae burned off recovered from the operation sufficiently to eat candy and to move about freely, but they were far from being normal. These lived from 1 to 11 days. When tested with the three essential oils, peppermint, thyme, and wintergreen, they responded readily. Their general response was to move slightly and vibrate the stubs of their antennae; one rubbed a leg against the abdomen and 3 moved their heads quickly. Average reaction time for oil of peppermint 3.5 seconds, for oil of thyme 4.5 seconds, for oil of wintergreen 4.3 seconds, and for all three oils 4 seconds. Frequently, when the antennae were cut or burnt off, the insects were placed on the table and tested with these odors. They often moved away from the odors but generally did not react to odors or anything else and often scarcely moved even when touched with a pencil.

In previous experiments bees cleaned off any substance put on their antennae. To prevent this the tarsi of their front legs

were burned off with a red-hot needle, removing their antennal cleaners. About one-fourth of the bees so mutilated died within 12 hours, but the remainder appeared quite normal in every other way. On the second day eleven joints or three of the flagellum of each antenna was coated with liquid glue. Since the antennal cleaners were removed, these bees could not remove the thick coating of glue. They were quite abnormal and most of them did not live long. However, after gluing the flagella of many bees, 21 were finally obtained that were as normal and they responded to the odors of the above oils without failure. The general response was either to arise and move away quickly or to vibrate their antennae. Often when a bee had fallen down apparently lifeless the odor was placed underneath it; it arose almost instantaneously and moved away quickly. After 3 or 4 days all the surviving bees had succeeded in cleaning their antennae but most of them died before this time. However, some lived 12 to 14 days. The average reaction time to oil of peppermint was 3.1 seconds, to oil of thyme 3.3 seconds, to oil of wintergreen 2.8 seconds, and for all three oils 2.9 seconds.

To ascertain if the odor of the glue itself affected these results in any way, glue was placed on the top of the thorax. This dried in a few minutes and the bees were not able to get it off. They were entirely normal and were tested with the foregoing oils. The average reaction time to oil of peppermint was 2.4 seconds, to oil of thyme 2.8 seconds, to oil of wintergreen 3.1 seconds, and for all three oils 2.7 seconds. The workers tested were 19 in number.

From all the experiments performed on the antennae of bees it is evident that when these appendages are mutilated in the slightest degree, the bees are never entirely normal, even though they apparently recover from the effect of the shock. The greater the number of joints removed or covered, the greater the abnormality. Furthermore, there is no reason to assume the presence of the olfactory organs on the antennae, because when these appendages were burned off, the general average of the reaction time for the three oils was 4 seconds; when glued, 2.9 seconds; and when un mutilated, 2.6 seconds. This slight in-

crease in time can certainly be attributed to the abnormal condition of the bees, and these results indicate that the olfactory organs are located elsewhere. At most it can be claimed only that the antennae may assist in the receiving of odor stimuli.

#### MAXILLAE AND LABIAL PALPI

Various observers claim that the palpi of insects are the seat of the olfactory organs. In the bee the maxillary palpi are almost wanting, but the maxillae and labial palpi possess pore-like sense organs. These appendages were cut off at the base. When introduced into observation cases, bees so treated appeared quite normal in all other respects and when strange bees were put among them they lost no time in attacking the strangers. Nevertheless, they certainly were not completely normal, for they lived only from 3 hours to 4 days, with 24 hours as an average. When tested with odors, they gave responses similar to those of uninjured bees. The reaction time was as follows: Oil of peppermint 2.2 seconds, oil of thyme 3.7 seconds, oil of wintergreen 4 seconds, honey and comb 4.4 seconds, pollen 5.8 seconds, and leaves and stems of pennyroyal 3.9 seconds. This gives a general average of 4 seconds, whereas for the same odors with un mutilated bees the general average was 3.4 seconds.

#### PROBOSCIS

Several writers have described sense organs on the proboscis of insects. To determine whether these have an olfactory use, the proboscides of 36 workers were cut off close to the base. These bees seemed quite normal in most respects and many of them even tried to eat candy, but of course they could not accomplish much without this appendage. They lived only 7 hours on an average. The average reaction time to oil of peppermint was 2.6 seconds, to oil of thyme 3 seconds, and to oil of wintergreen 3.2 seconds. This gives a general average of 2.9 seconds, while for the same odors with un mutilated bees the average was 2.6 seconds. Twenty-two workers were tested. We can probably attribute this difference of 0.3 seconds to the abnormality of the mutilated bees.



## MANDIBLES

Janet ('11b) describes a sense organ in the mandible of the honey bee which he thinks may have an olfactory function. To ascertain this experimentally, the mandibles of 30 workers were amputated close to the base. These bees appeared completely normal and did not fight each other. They lived from 5 hours to 15 days, with 7 days as an average. The average reaction time to oil of peppermint was 2.6 seconds, to oil of thyme 3.3 seconds, to oil of wintergreen 4.6 seconds, to honey and comb 7.7 seconds, to pollen 6.2 seconds and to leaves and stems of pennyroyal 4.7 seconds. These give a general average of 4.8 seconds whereas the average for the same odors of unamputated bees was 3.4 seconds. We may attribute the slight difference in time either to the injury caused by the amputation, or to the fact that the mandibles help to perceive odors or to both. The bees tested were 20 in number.

## BUCCAL CAVITY

Huber (1814) states that the seat of the organs of smell in the honey bee is the buccal cavity, whereas Wolff (1875) discovered some sensilla on the epipharynx of the same insect. The buccal cavity is in the mouth cavity and forms a portion of its floor. To determine whether this cavity has anything to do with olfaction, Huber's experiment of filling the mouth cavity with flour paste was repeated. With the aid of a small pencil brush the mouth cavities of 25 workers were thus filled. When the paste had become perfectly dry, the bees were put into observation cases. They seemed otherwise entirely normal and all tried to eat candy although some were unable to move their probosces on account of the hard paste. They lived from 2 hours to 16 days, with  $7\frac{1}{2}$  days as an average. However, by the end of the fourth day the paste had come out of their mouths. The average reaction time to oil of peppermint was 2.55 seconds, to oil of thyme 2.75 seconds, and to oil of wintergreen 2.75 seconds. This gives a general average 2.68 seconds. The average for

the odors with normal bees was 2.64 seconds. Twenty workers were tested. It would seem that neither the buccal cavity nor the epipharynx has anything to do with olfaction.

#### MORPHOLOGY OF OLFACTORY PORES

In 1857, 1860 Hicks described for the first time some peculiar structures found on the bases of the wings and legs of insects. He called them 'vesicles' and suggested that they have an olfactory function. From the following description of the morphology of these structures it will be seen that the word 'vesicle' is less appropriate than the word 'pore.' Since they have an olfactory function in the honey bee (pp. 333-341) they may be called 'olfactory pores.'

#### DISPOSITION

In studying the distribution and number of the olfactory pores of the honey bee, workers and drones just emerged from the cells were selected on account of their lighter color, but since queens at this stage were not available at the right time, old dark-pigmented ones were used. The three legs from one side of 6 individuals each of workers, queens, and drones and the three legs from the other side of 9 of these 18 bees were examined under a high-power lens. The wings from both sides of 8 workers, 8 drones, and 4 queens and the stings of 15 workers and of 9 queens were likewise examined. In all, 81 legs, 80 wings, and 24 stings were searched for pores.

The wings have upper (dorsal) and lower (ventral) surfaces and the legs have inner and outer surfaces. The inner side of the leg faces the bee's body, and the outer side is directed from the body except in the case of the front legs, which are directed forward and the sides are therefore reversed. These pores are found in groups, and for convenience in studying and describing them the groups on each side may be numbered from 1 to 21. The first five of these groups are found at the bases of the wings, groups 6 to 18 inclusive on the legs, and the remaining three groups on the sting (figs. 1, 2, and 14, A). The numbers of the

## ABBREVIATIONS

<i>1A</i> , <i>3A</i> , first anal vein, third anal vein	<i>Ob</i> , oblong plate of sting
<i>AgCyt</i> , deeply staining cytoplasm	<i>Pg</i> , peg, club, or sensilla trichonica
<i>Art</i> , articulation	<i>Por</i> , pore
<i>ArtCh</i> , articular chitin	<i>PorAp</i> , pore aperture
<i>ArtM</i> , articulation membrane	<i>PorApHr</i> , pore aperture hair
<i>1Ax</i> , <i>2Ax</i> , <i>3Ax</i> , first axillary, second axillary, third axillary	<i>PorB</i> , pore border
<i>AxC</i> , axillary cord	<i>PorBHr</i> , pore border hair
<i>BC</i> , body cavity	<i>PorCl</i> , pore canal
<i>BGL</i> , alkaline gland of sting	<i>PorHr</i> , pore of hair
<i>BlCor</i> , blood corpuscle	<i>PorPl</i> , pore plate, pore canal, or sensilla placodea
<i>BlSin</i> , blood sinus	<i>PorW</i> , pore wall
<i>Brb</i> , barb	<i>PorWHr</i> , pore wall of hair
<i>BrbHr</i> , barbed hair	<i>PPg</i> , pit pegs, champagne-cork organs or sensilla steloconica
<i>C</i> , costa	<i>PsnCl</i> , poison canal of sting
<i>Ch</i> , chitin	<i>PsnSc</i> , acid poison sac of sting
<i>ChL</i> , chitinous layer	<i>Qd</i> , quadrate plate of sting
<i>ChM</i> , chitinous marking	<i>R</i> , radius
<i>ChTh</i> , chitin of thorax	<i>R+M</i> , radius and media united
<i>Con</i> , cone	<i>Sar</i> , sarcolemma
<i>ConnT</i> , connective tissue	<i>SarNuc</i> , nucleus of sarcolemma
<i>Cu</i> , cubitus	<i>SC</i> , sense cell
<i>Cyt</i> , cytoplasm	<i>SCG</i> , sense cell ganglion
<i>FfL</i> , Forel's flask or sensilla ampullacea	<i>Scl</i> , scutellum of mesotergum
<i>Gr</i> , groove	<i>SCNuc</i> , nucleus of sense cell
<i>Hr</i> , hair	<i>SCNuc<sup>1</sup></i> , nucleus of sense cell without cell wall
<i>Hyp</i> , hypodermis	<i>SCNucl</i> , nucleolus of sense cell
<i>HypCW</i> , hypodermal cell wall	<i>SF</i> , sense fiber
<i>HypNuc</i> , hypodermal nucleus	<i>Sh</i> , shaft of sting
<i>HypNuc<sup>1</sup></i> , hypodermal nucleus that has formed a hair	<i>ShB</i> , bulb of shaft of sting
<i>IXS</i> , thick membranous lobe that overlaps sting	<i>SpHr<sup>1, 2, 3</sup></i> , three varieties of spinelike hairs
<i>Lct</i> , lancet	<i>StnPlp</i> , palpus of sting
<i>M</i> , muscle	<i>1Tar</i> , <i>2Tar</i> , <i>3Tar</i> , <i>4Tar</i> , <i>5Tar</i> , first to fifth tarsal joints
<i>MB</i> , muscle bundle	<i>Tg</i> , tegula
<i>MBNuc</i> , nucleus of muscle bundle	<i>THr</i> , tactile hair or sensilla trichodea
<i>Md</i> , median plate	<i>Tn</i> , taenidia of trachea
<i>MFL</i> , mouth of flask	<i>Tra</i> , trachea
<i>N</i> , nerve	<i>TraNuc</i> , nucleus of trachea
<i>NB</i> , nerve branch	<i>Tri</i> , triangular plate of sting
<i>Neu</i> , neurilemma	<i>1 to 21</i> , groups 1 to 21 of the olfactory pores
<i>NeuNuc</i> , nucleus of neurilemma	
<i>NeurNuc</i> , neuroglia nucleus	
<i>NF</i> , nerve fiber	
<i>NkFL</i> , neck of flask	

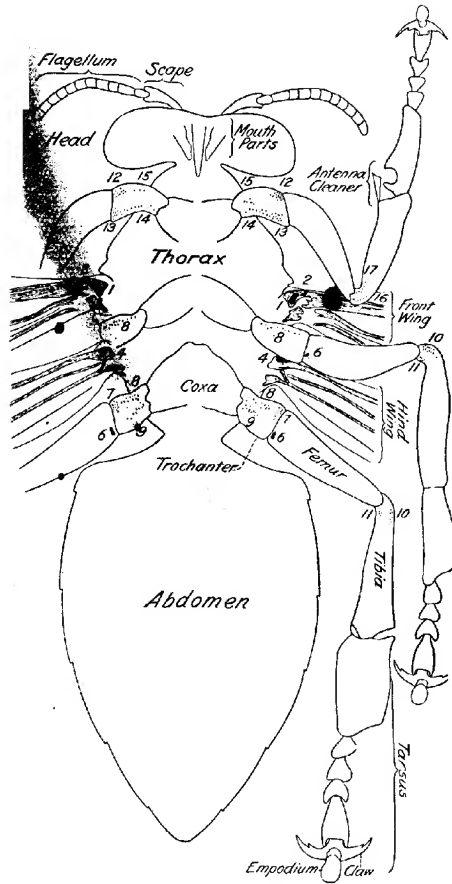


Fig. 1 Diagram of ventral view of a worker bee, showing the location of the different groups of olfactory pores as indicated by the numbers.

groups on the legs apply to similarly placed groups on all the legs, but the groups on the front and hind wings are seemingly not homodynamous and are given different numbers. If a group has been found to be always similarly placed and always

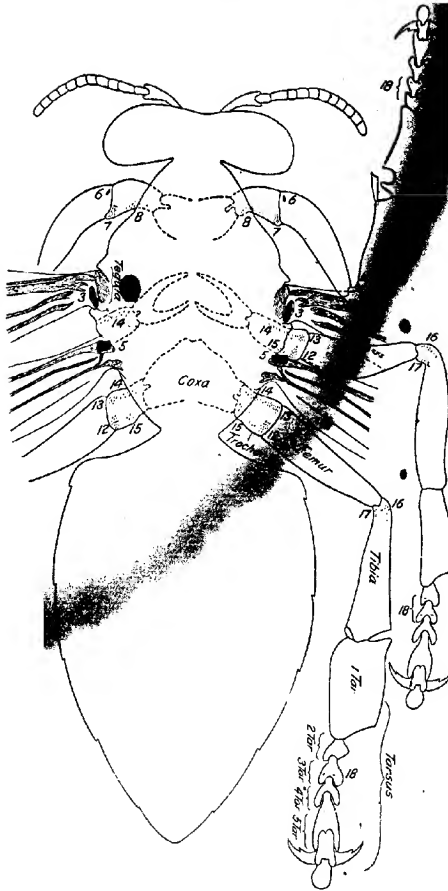


Fig. 2 Diagram of dorsal view of a worker bee, showing the location of the different groups of olfactory pores as indicated by the numbers.

to have the same general shape, it may be regarded as constant and if the pores are close together it may be regarded as definite; otherwise it may be considered as inconstant and scattered.

A worker bee is most suitable for study, the pores on the wings, third leg, and the sting of specimen No. 15, which has been shown (figs. 1 and 2), will be described in detail. The differences in the other legs of this specimen and the variations found in the three castes will then be discussed.

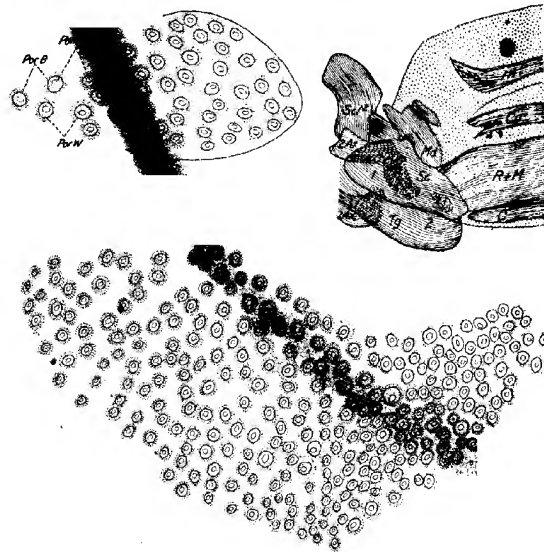


Fig. 3 Ventral view of base of front wing of a worker bee, showing groups 1 and 2 of olfactory pores.  $\times 45$ .

Fig. 4 External view of group 1 of olfactory pores of a worker bee.  $\times 465$ .

Fig. 5 External view of group 2 of olfactory pores of a worker bee.  $\times 465$ .

Groups 1 and 2 (figs. 1 and 3) lie on the lower surface of the front wing. Group 1 occupies almost a central position on the subcosta, while group 2 lies near the anterior and distal margin (fig. 3, Sc.). These groups are somewhat triangular in shape, with their apices facing each other, and the concave edge of group 1 always faces the posterior margin of the subcosta. A light band (represented by a line in fig. 5), surrounds the distal

end of group 2. In groups 1 and 2 there are 281 and 281 pores respectively; in the former group (fig. 4) the pores are close together while in the latter (fig. 5) they are more scattered. The pores vary considerably in size; in group 1 most of them are small, with the diameter of the largest three times that of the smallest, while in group 2 almost all of them are comparatively large, with the diameter of the smallest one-half that of the largest. In group 1 scarcely any rows are discernible, but in group 2 the rows are more sharply defined. The openings (or Ap) in the pores are usually round, although several are oblong, and the long diameter of the oblong pores is more or less parallel with that of the group.

Group 3 is found on the upper surface of the front wing (fig. 2) and occupies nearly the entire surface of the median plate (fig. 6, *Md*), leaving only a narrow margin on all sides. This group is long and slender, with its tapering end pointing toward the proximal and posterior margin of the subcosta (*Sc*). Its pores are considerably scattered, although they lie in more or less definite rows (fig. 7). The pores are about equal in diameter and the diameter of the largest is never more than twice that of the smallest. In this group there are 174 pores, nearly all of which have round apertures.

Group 4 is present on the lower surface of the hind wing (fig. 8) and covers most of the anterior half of the union of the radius and media (fig. 8, *R + M*). Its distal end is the more pointed and its pores are arranged in irregular rows (fig. 9). There are 83 pores of about equal size.

Group 5 lies on the upper surface of the hind wing (fig. 2) and occupies nearly all of the surface where the radius and media unite (fig. 10, *R + M*) and extends slightly over the subcosta (*Sc*). The proximal and narrower end of the group points directly toward the proximal end of the subcosta. There are 209 pores, which can scarcely be said to lie in rows (fig. 11). Most of the pores are small, with the diameter of the largest three times that of the smallest.

Group 6 is a double group having the shape of a figure 8, located at the proximal end of the femur (fig. 1) on the outer

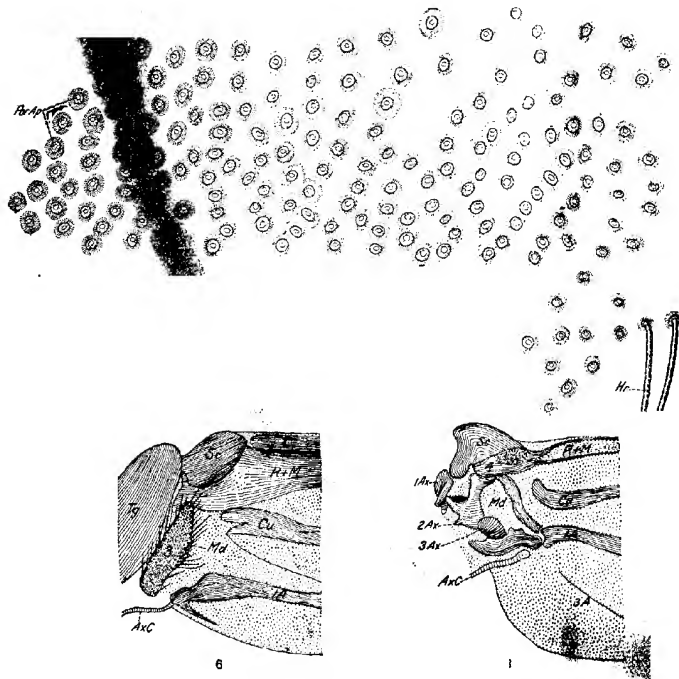


Fig. 6 Dorsal view of base of front wing of a worker bee, showing group 3 of olfactory pores.  $\times 45$ .

Fig. 7 External view of group 3 of olfactory pores of a worker bee. The distal end with two hairs is placed beneath the remainder of the group.  $\times 465$ .

Fig. 8 Ventral view of base of hind wing of a worker bee, showing group 4 of olfactory pores.  $\times 45$ .

surface near the posterior margin. The smaller portion of group 6 lies nearer the posterior edge of the leg (fig. 12, A) and has 9 pores, none of whose openings lies with its long diameter exactly transverse to the long axis of the leg. The other portion of the group has thirteen pores, and the long diameter of most of its openings are exactly transverse to the long axis of the leg. The diameter of the largest pore is about twice that of the smallest.



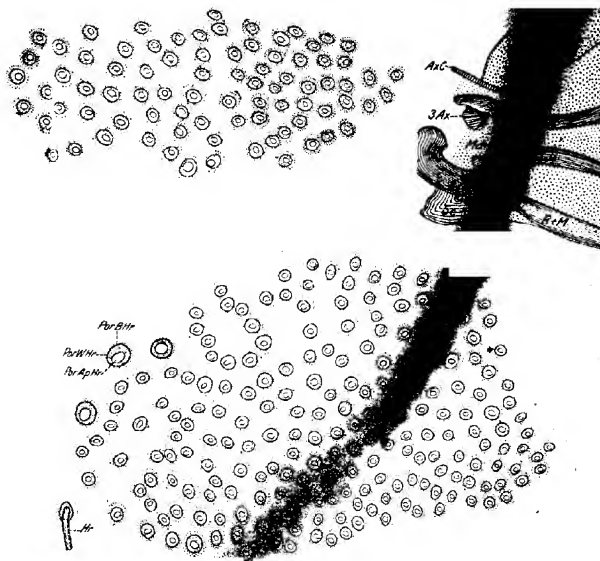


Fig. 9 External view of group 4 of olfactory pores of a worker bee.  $\times 465$ .

Detail of base of hind wing of a worker bee, showing group 5 pores.  $\times 45$ .

External view of group 5 of olfactory pores of a worker bee.  $\times 465$ .

The majority of the pores on the legs are slightly oblong and have openings which as a rule have their long diameters more or less transverse to the long axis of the leg.

Groups 7, 8, and 9 lie on the outer side of the trochanter (fig. 10). Group 7 has 16 pores and lies at the distal end near the anterior margin, group 8 extends along the anterior margin from group 7 almost to the articulation of the trochanter with the coxa, and group 9 extends from the proximal end along the median line two-thirds the distance to the femur. In all three groups the diameter of the largest pore may be three times that of the smallest. The pores of groups 7 and 9 (figs. 12, B and D) have their long diameters directed obliquely across the leg, while in

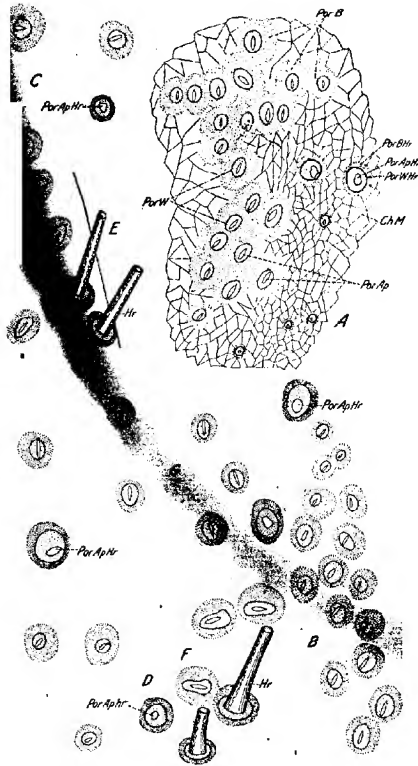


Fig. 12. External view of olfactory pores as they appear on the third leg of a worker bee,  $\times 465$ . A is group 6; B, group 7; C, three olfactory pores and a hair pore (*PorApHr*) from group 8; D, three olfactory pores and a hair pore from group 9; E and F, a few olfactory pores and hairs from groups 10 and 11; G, six olfactory pores and a hair pore from group 12.

group 8 (fig. 12, C) most of the long diameters are parallel to the long axis of the trochanter.

Groups 10 and 11 are found at the proximal end of the tibia (fig. 1) on the outer side. Group 10 is near the anterior edge and group 11 near the posterior. The pores of these groups are

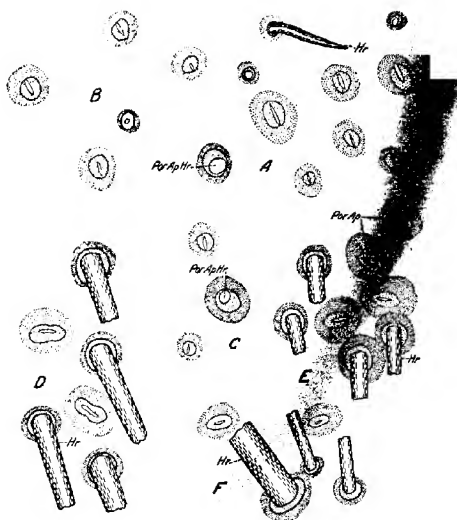


Fig. 13 External view of olfactory pores and some spinelike hairs as they appear on the third leg of a worker bee,  $\times 465$ . A, from group 13; B, from group 14; C, from group 15; D and E from groups 16 and 17 respectively; F, from group 18, the two pores are drawn twice too close together.

largest of all and they do not vary greatly in size. They are oblong and the long diameters are in some cases oblique and in others transverse to the long axis of the tibia (fig. 12, E and F). They lie in rather straight rows, along the long axis of the leg.

Groups 12 to 15 lie on the inner side of the trochanter (fig. 2). Groups 12 and 13 are located at the distal end, with group 12 near the posterior edge and group 13 near the anterior. Group 14 lies on the inner side in a position near the anterior edge similar to that of group 8 on the outer side. Group 15 lies near the posterior margin extending the full length of the trochanter (figs. 12, G and 13, C). In these preparations occasionally one end of the slits extends outside the pore wall (*PorW*). This may be due to the treatment with caustic potash.

Groups 6 and 17 (figs. 2, 13, D and E) are similar to groups 10 and 11 except that they are on the inner side of the proximal end of the tibia.

Group 18 consists of two or three pores as large as those on the tibia, which are found on the ventral side of the second and third tarsal joints (fig. 2). These two pores and three hairs are represented in figure 13, F, although in the drawing the relative distance between the pores is reduced one-half.

Group 19 is located on the shaft of the sting (fig. 14, A) and its pores are greatly scattered, but most of them occur about midway between the two ends. A few are present on the tip end of the shaft (fig. 14, A, *a*), while three are located at the other extreme end (fig. 14, A, *b*). This group contains 44 pores. Figure 14, B, shows the pores on the tip of the shaft with a high magnification. The canal (*PorCl*) passes from the apertures into the body cavity (*BC*) of the shaft. Snodgrass ('10) presents a similar drawing, although he represents too many pores near the tip end of the shaft. Figure 14, C, represents a pore from the tip end of the shaft at *a* and figure 14, D, two pores from the point marked *e* in figure 14, A, with a still higher magnification. Figure 14, E, represents five pores from the point marked *c*.

Group 20 occurs on the tip end of each lancet (fig. 14, A, *Brb*). Here there are 10 pores and an equal number of barbs, the opening of each pore being found at the base of a barb. With the highest magnification it is plainly seen that the canal of the pore runs into the body cavity of the lancet (fig. 14, F, *BC*).

Group 21 lies on each lancet just behind the lowermost part of the bulb of the shaft (fig. 14, A, *d*). It has 10 pores,<sup>2</sup> which vary considerably in size (fig. 14, G).

The following differences were found in the various groups of pores on the first and second legs of specimen No. 15: On each of these two legs group 6 is a single group and has only 18 pores. It does not lie quite so close to the posterior edge as on the third leg. Group 7 is not found on the second leg, but on the first it

<sup>2</sup> The drawing (fig. 14, G) shows only eight of these pores.



The specimen No. 15, groups 1 to 6 are strictly definite and constant. Group 7 is not always present, but it is definite when present. Group 8 and groups 10 to 18 are always present, and are scattered, groups 8, 14, and 15 being scattered most, and they vary slightly in position. Group 9 is found only on the third leg, and it is scattered more than any other group.

#### *Variation*

The groups of pores differ in shape and size in the different legs of the same individual, and different individuals show slight variation in homologous groups. The following account gives the variations observed in the specimens examined.

*Wings of workers.* Due to the wrinkled and folded condition of many of the wings of all three castes, some of the groups were not discernible and the pores of many groups could not all be counted. For this reason the data for all the wings examined can not be presented. However, all five groups are invariably seen to be present when the preparations are good. These groups vary considerably in number of pores. Group 1 varies from 230 to 312, with an average of 266 for 10 wings. In three cases this group had its broader ends divided into two branches. Group 2 varies from 47 to 58, with an average of 53 for 10 wings. Group 3 varies from 156 to 192, with an average of 172 for 10 wings. Group 4 varies from 76 to 86, with 81 as an average for 15 wings. Group 5 varies from 171 to 209, with 189 as an average for 9 wings. Thus on the front wing there is an average of 485 pores and on the hind wing 270, making 755 pores on both wings of one side or 1510 on all four wings.

*Legs of workers.* In examining nine legs of the third pair belonging to six workers, No. 6 is always present and it is double in each case. In number of pores the smaller group varies from 8 to 10, and the larger group from 10 to 13, giving an average of 20 pores for No. 6. On the second leg this group is present on only seven of the nine legs examined; it is single in each case, but on three legs it is found on the inner instead of the outer surface. Its pores vary from 6 to 20, with 18 as an average.

This group is always present on the outer side of the third leg; it may or may not be double, and its pores vary from 14 to 20.

Group 7 of the third leg is always present, and its pores vary in number from 14 to 16. On the second leg this group may be definite or scattered, but usually has about 15 pores. This group on the first leg is always definite, with about 15 pores.

The pores of groups 8 and 9 are always considered scattered and usually these two groups can not be separated. They are therefore not counted separately. Group 9 occurs only on the third leg and is sometimes not found even on that leg. On the third leg the number of pores of both groups varies from 7 to 21. On the second leg group 8 varies from 6 to 35 and on the first leg from 6 to 16 pores.

Groups 10 and 11 may exist as two separate and scattered groups or as one scattered group. On the third leg the pores of these two groups combined vary in number from 13 to 26, with about 16 as an average; on the second leg from 6 to 20, with 14 as an average; on the first leg from 4 to 20, with about 12 as an average.

Groups 12 to 15 are usually present on all three legs. Groups 12 and 13 are relatively constant in position and number of pores, while groups 14 and 15 are much scattered and vary considerably in number of pores. Group 12 usually consists of 15 to 16 pores, while No. 13 consists of a few less, although its pores often can not be separated from those of No. 14. The combined pores for all four groups on the third leg vary in number from 24 to 48; for the second leg, from 13 to 44, with none on the inner side of one leg, but with more pores than usual on the other side of this leg; for the first leg, 32 to 47.

Groups 16 and 17 are very similar to groups 10 and 11 except that they do not have so many pores and that on half the legs one of them is either absent or united with the other group. Their combined pores vary from 3 to 15, with about 10 as an average.

Group 18 is discernible in every case where the ventral sides of the tarsal joints directly face either cover glass, although only about a third of the tarsi happen to be turned in such a position. Usually two large pores are present on the third tar-

sal joints. Sometimes only one, either on the second or third joint. These pores occur in the clear spot where there are no hairs and just above the nearest place of articulation, one on either side of the median line.

For the worker legs examined, the total number of pores found on the outer side of all three legs on one side varies from 153 to 211, and on the inner side from 107 to 170, giving a variation from 260 to 372 for both sides, or an average of 329.

*Stings of workers.* The pores on both sides of the stings of 15 workers were counted, and in location and number they vary greatly in group. There are usually only a few at the tip of the shaft, while most of them lie at the distal end of the bulb (fig. 14, A, c) and only occasionally are there any pores at the point marked b. Group 20 is constant and definite, with a pore at the base of each of the 10 or 11 barbs. Group 21 is also constant and definite, but may vary in number of pores from 6 to 12, with 9 as an average. Sometimes there are 2 or 3 scattered pores on the oblong plate (fig. 14, A, Ob). For the 15 stings examined, the number of pores on both sides varies from 84 to 128, with an average of 100.

On the four wings, six legs, and the sting of an average worker bee there are 2268 pores.

*Wings of queens.* On the wings of queens group 1 varies from 200 to 249 pores, with an average of 225; group 2, from 42 to 51, with 47 as an average; group 3, from 141 to 156, with 148 as an average; group 4, from 61 to 85, with an average of 72; and group 5, from 144 to 176, with 163 as an average. To obtain these numbers five examples each of groups 1 to 3, 6 of No. 4, and 3 of No. 5 were counted. On the front wing there are 420 and on the hind wing 235 pores, making an average of 1310 pores for all four wings of a queen.

*Legs of queens.* On the legs of queens group 6 is double in every instance and it is always present on the outer side in all three legs. On the third legs it varies in number of pores from 15 to 20, with 18 as an average; on the second legs, from 16 to 21, with an average of 17; on the first legs, from 15 to 19, with an average of 17.



On the third and first legs group 7 occurs three out of nine times and on the second legs four out of nine times. The number of pores it varies from 6 to 16, with about 12 as an average.

Groups 8 and 9 are either totally absent or when present they consist of only a few pores which are widely scattered. When present the pores of the two groups combined vary from 2 to 19.

Groups 10 and 11 are both always present on the third legs, their number of pores varying from 9 to 19. On the second leg one of these groups is absent in four instances and their pores vary in number from 1 to 13. On the first legs one group is missing twice, their pores here varying from 5 to 13.

Groups 12 to 15 are more scattered than in the workers, and the pores are fewer in number. Only occasionally are groups 12 and 13 more or less definite, while in the workers they are quite definite. The total number of pores for all four groups varies from 2 to 41.

On the third leg either group 16 or 17 is always absent, and in one case both groups are missing. Their total number of pores varies from 0 to 9. On the second leg both groups are usually present, with their pores varying in number from 4 to 20. On the first leg one and occasionally both groups are absent, their number of pores varying from 0 to 12.

Group 18 was never discernible in the queens.

Among the nine sets of legs of queens examined the pores for the outer side vary in number from 90 to 150 and for the inner side from 55 to 117, giving a variation in number from 172 to 262, with an average of 225 pores for both sides of three legs.

*Stings of queens.* An examination of the pores on both sides of nine queen stings showed that their disposition is similar to that of the worker stings. The combined number of pores for both sides of these stings varies from 83 to 111, with 100 as an average.

From a count of the pores on the four wings, six legs, and stinging an average queen, therefore, is found to possess 1860 of these organs.

*Wings of drones.* On the wings of drones group 1 varies in number from 310 to 325, with 316 as an average; group 2, from

53 to 64 with 64 as an average; group 3, from 217 to 280, with an average of 236; group 4, from 108 to 148, with an average of 115; group 5, from 240 to 290, with an average of 268. To obtain these figures the pores were counted in group No. 1 in 5 individuals, in group No. 2 in 10, in group No. 3 in 11, in group No. 4 in 14 and in group No. 5 in 6. For the front wing there is an average number of 616 and for the hind wing 383, making an average total of 1998 pores for all four wings of a drone.

*Legs of drones.* On the legs of drones group 6 is constant and is always double on the third legs; its pores vary in number from 17 to 25, with 20 as an average. On the second legs group 6 is absent three times out of nine, and once it occurs on the inner instead of the outer side. When present here it is always single and its pores vary in number from 9 to 16. On the first legs this group is missing only once. It is always double and its pores vary in number from 17 to 20.

Group 7 is missing once on the third legs and twice on the second and first legs. For the third legs its pores vary from 11 to 19, for the second legs from 16 to 23, and for the first legs from 17 to 21.

Groups 8 and 9 are much scattered and are difficult to separate. Group 8 is almost always and 9 is only occasionally present. Their combined pores vary from 0 to 27 for any leg.

On the third legs groups 10 and 11 almost always exist as one scattered group, with pores varying in number from 13 to 22. On the second legs both groups are generally present, their pores varying in number from 9 to 24. Both groups are always found on the first legs. Here their pores vary in number from 10 to 18.

Groups 12 to 15 are invariably present and are considerably scattered, so that it is impossible sometimes to separate them. Group 12 is more or less definite, while group 13 always merges with group 14. The total number of pores of all four groups for the third legs varies from 11 to 49; for the second legs, from 5 to 46; for the first legs, from 12 to 45.

Groups 16 and 17 are similar to groups 10 and 11 on the outer side and in number of pores they have about the same variation.

Group 18 was discernible on all the tarsi. About two and sometimes three large pores were present on the third tarsal joints.

As the drone has no sting, the fleshy external surface of the penis of several drones was carefully examined. The surface of the penis is always covered with hairs but no pores were found.

For the nine sets of legs, on the outer side there is a variation from 154 to 201 and for the inner side from 117 to 148, making a total variation from 265 to 349 for both sides, with an average of 303 pores for both sides of three legs. On all six legs and all four wings the drone possesses an average of 2604 pores.

The thorax, abdomen, head, head appendages—in fact all parts of the chitinous skeleton—of several workers, queens, and drones were examined. Of these appendages only the mouth parts contain porelike organs similar in external appearance to these pores. They are not included in this paper, although they may be olfactory pores.

#### *Summary*

For workers, queens, and drones these pores are found on the sides of the bases of all four wings, scattered rather profusely on both sides of the trochanter of all the legs, on the outer side of the proximal end of each femur, on both sides of the tibia at the proximal end, on the second and third tarsal joints, and considerably scattered on the shaft and lancets of the sting. The five groups of pores on the wings are always present, those on the femur, tibia, and sting are generally present, while those on the trochanter and tarsal joints may be partially or almost wholly absent. Generally speaking, all of the 21 groups are nearly always present, are constant in position, but vary considerably in the number of pores.

To compare the total number of pores on one side of an individual with the total number on the other side of the same specimen, or with either side of different individuals, it has been ascertained that the variation in total number of pores is considerable.

This number is nearly the same for workers and drones, but is much less for the queens.

As a rule each group contains at least a few more pores on the drone than on the worker or the queen, and the trochanters of the drone have many more pores than do those of the queen and several more than do those of the worker. Nevertheless, since a drone has no sting and consequently lacks the organs belonging to it, the total number of pores is correspondingly lessened. The individual variations for the total number of pores of all three castes are slight.

The number of pores for each group on the wings for the three castes varies, while on the legs of all three castes the total number of pores is relatively constant. In all three the size of the groups and even the dimensions of the pores are relatively the same. Hence, since drones have the most pores and the queens the least, the pores in the former are the most densely grouped and those of the queens the least.

The following table shows the average number of olfactory pores on all four wings, on all six legs, and on the sting of the three castes of the honey bee and also the average total number of pores for each class.

TABLE 1

*Average number of olfactory pores on the wings, legs and sting of the honey bee*

CLASS	NUMBER OF PORES ON ALL FOUR WINGS	NUMBER OF PORES ON ALL SIX LEGS	NUMBER OF PORES ON STING	TOTAL NUMBER OF PORES
Drone.....	1998	606		2604
Worker.....	1510	658	100	2268
Queen.....	1310	450	100	1860

Since the queen possesses an average of 1860 pores, the worker an average of 2268, and the drone an average of 2604, it seems probable that whatever function they perform this is best developed in the drone and least in the queen, in proportion to the average number of pores.

Various kinds of sense organs exist on the antennae and mouth parts of the bee. Neither the external appearance nor the internal structure of any of the antennal organs has the slightest

resemblance to these olfactory pores. Janet ('11a) speaks of a chordotonal organ at the base of the antenna in the honey bee, but it has no resemblance to the olfactory pores. Hildebrand ('60) also found some pores on the proboscis and palpi of the honey bee and of other insects. Breithaupt ('86) also described some pores found on the base of the bee's proboscis. The drawings of the last two authors are not clear, so far as the anatomy of the pores is concerned. These same pores are found not only at the base of the proboscis but elsewhere on this appendage and also on the labial palpi and maxillae. Their superficial appearance is similar to the olfactory pores. Briant ('84) describes sense hairs on the proboscis and maxillae of the honey bee; these hairs, however, have nothing in common with the olfactory pores. Packard ('89) and Nagel ('97) find hairlike sense organs in the buccal cavity of various insects, but their structure does not resemble that of the olfactory pores. In all probability none of these organs on the head appendages is homologous in structure and function to the olfactory pores, except those on the mouth parts, but a more critical study of them would be required before anything definite could be stated concerning them. While it would not be surprising to find the olfactory pores in places other than those already named, nevertheless it is believed that nearly all, if not all, have been found.

#### STRUCTURE

##### *External structure*

When examined under a low-power lens the olfactory pores may be easily mistaken for hair sockets from which the hairs have been removed. When more carefully observed, even under a low-power lens, a striking difference in external form is seen. The five groups on the wings are quickly detected, but those on the legs and sting are not so readily found. If the preparation is dark, the pores appear as small bright spots, when a strong transmitted light is used. Looking at these areas under a high-power lens each one appears to have a dark boundary, or pore wall (fig. 5, *PorW*). Near the center of this boundary is a trans-

parent, which may be round, oblong, or slitlike. On the wings the transparent spots are always round or oblong (fig. 5, *PorAp*) and never slitlike as on the legs (fig. 12, A, *PorAp*). By slowly focusing down on this transparent area it appears to diminish in size and at the lowest level is a transparent spot, which is perfectly round on the wings and legs. This spot is an opening in the chitin, the pore aperture (*PorAp*). If the specimen examined is light, the dark pigmented pore border is more noticeable. On the wings this border is narrow (fig. 5, *PorB*) while on the legs, where the pores are not so close together, it is wider and when the pores are near each other the borders (fig. 12, A, *PorB*) merge and appear more conspicuous.

The chitin between the pores on the legs is marked, as elsewhere, with light colored lines (fig. 12, A, *ChM*). These markings on the chitin are never present between the pores on the wings but occur everywhere outside the groups. These markings on the chitin have been omitted in all the drawings except figure 12, A.

In comparing a hair socket with an olfactory pore the following differences are noticed; the largest pore is never as large as the largest socket, and the boundary of the hair socket (fig. 12, A, *PorW Hr*) is always much darker and heavier, its aperture is never slitlike, and even if round or oblong it is ragged, showing where the hair has torn the edges when pulled out. As a rule the border of the hair socket (*PorB Hr*) and even the chitin inside the boundary are much darker than are comparable parts of the olfactory pore. The borders of the hair sockets are usually much narrower than are those of the pores. All these differences are fairly constant, but the sockets of the smallest of the spinelike hairs (fig. 15, *Sp Hr*) on the legs are almost identical in appearance with the smallest pores and they are distinguished only by the ragged condition of the sockets. In figure 15, on the trochanter, the various kinds of hairs and the pores which belong to groups 7, 8, and 9 are shown. The pores are shown with slitlike openings while the hair sockets have irregular rings round the sockets. If these pores were drawn with dark borders, as they really appear when seen through the microscope and as the

hair sockets are represented in this drawing, the accuracy of observation would be appreciated.

From the above it is seen that the superficial appearance of an olfactory pore is that of a small round or oblong light area that has a dark boundary and a round, oblong, or flask-like trans-

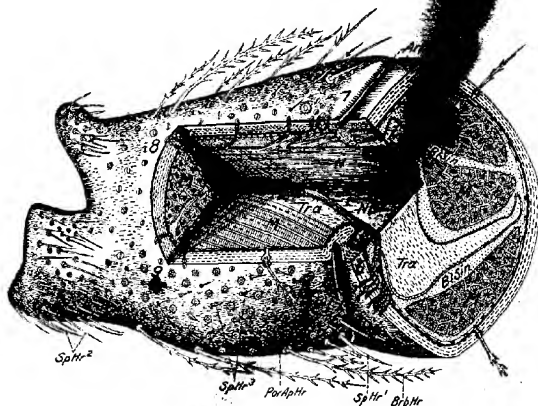


Fig. 15 Diagram of a longitudinal transverse view of the trochanter and a small portion of the femur from the third leg of a worker bee, showing the location of the olfactory pores on the outer surface as indicated by the numbers 6 through 100. The hairs and the anatomy of the leg are shown.

parent opening. Outside the boundary there is a comparatively dark border, and inside the border the chitin is very light in color. The aperture, according to its shape, may be likened to a round, slightly flattened, or considerably flattened funnel.

#### *Internal structure*

A typical olfactory pore of the honey bee is an inverted flask-like structure in which the bottom of the flask forms the external covering or chitinous layer (fig. 16, A, *ChL*) of the pore. This layer contains the pore aperture (*PorAp*). The neck (*NkFl*) of the flask is wide and the mouth (*MF*) is flaring. About two-thirds of the space at the bottom of the flask is occupied by a chitinous cone (*Con*). The cone is not separated from the walls

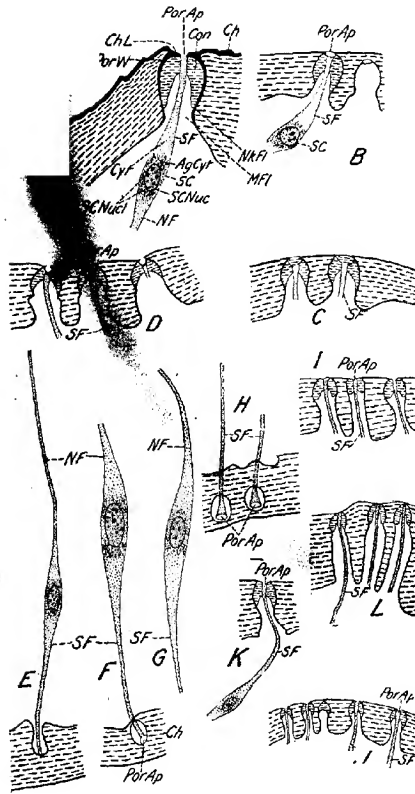


Fig. 16 Internal anatomy of olfactory pores of a honey bee,  $\times 700$ . A, a typical pore with its sense cell (SC) as actually seen in a section through the tibia. The longitudinal view of the pore aperture (PorAp) is shown. The edge of the chitin (ChL) and the walls of the flask (PorW) are drawn in solid black, since this part of the chitin stains more deeply. In other places in this and other drawings the chitin is represented by broken lines while the cones (Con) are shaded with fine, broken lines. The cytoplasm (Cyt) of the sense cells is represented by dots. ChL, chitinous layer; MFL, mouth of flask; NF, nerve fiber; SCNuc, sense cell nucleus; SCNucI, sense cell nucleoli; AgCyt, deeply staining cytoplasm. B, two pores and one sense cell from a cross section of the femur of the third leg; this section is one of the five included in figure 17



(*PorW*) of the surrounding flask, but it is evidently somewhat different in composition from the surrounding chitin (*Ch*), since it generally stains less deeply with iron hematein and eosin or with safranin and gentian violet. The apex of the cone is hollow and extends to the neck of the flask. The sense cell (*SC*) lies beneath the mouth of the flask. It is bipolar, long, and slender, and comparatively large. The peripheral pole or sense fiber (*SF*) of this cell runs into the hollow of the cone, pierces the bottom of the cone, and enters the lowest portion of the transparent pore aperture. The inner pole or nerve fiber (*NF*) of the sense cell joins a nerve cord. The nucleus (*SCNuc*), with its several dark staining bodies or nucleoli (*ScNucl*), is always conspicuous. While the cytoplasm (*Cyt*) in the poles is almost invisible, it is easily seen at either end of the nucleus, for here it stains rather deeply (*AgCyt*).

The flasks may be short and thick or long and slender, depending on the thickness of the chitin. In the legs they are generally short and thick (figs. 16, A-C, and 17) and have a short neck. Occasionally the basal portion of the flask is short, making a long neck whose walls are slightly evaginated (fig. 16, D). In the wings and sting the basal portion of the flask is short, but the neck is usually long and narrow (fig. 16, I-K; figs. 19-21, 23), and its walls rarely have a small evagination. In the femur and tibia the pores never pierce the thickest portion of the chitin, but in the trochanter, wings, and sting this may or may not be true.

The cone is always present. Its size and shape depend on the size and shape of the basal portion of the flask. With safranin

and passes through group 6; the circles at the top represent the slits cut cross-wise. C, two more pores from group 6, but here the slits are shown in longitudinal section. D, three pores from trochanter of third leg. E, F, two sense cells from two different sections of group 2 on the front wing; the chitin is cut obliquely. G, a sense cell from group 5 on the hind wing. H, two sense fibers entering pore apertures belonging to group 2 from front wing; the chitin is cut obliquely. I, three pores from group 3 from front wing entering pore apertures. J, five pores from group 4 on the hind wing, showing how sense fibers enter pore apertures. K, a typical pore and a sense cell from group 5 on hind wing. L, three pores and their sense fibers from a cross section of the tegula. Drawn with the aid of a camera lucida.

it invariably stains less deeply than the surrounding chitin, but with hematoxylin there is scarcely any difference in coloration.

Since the pore apertures are so small only occasionally does the microtome knife pass through the lowest part of the aperture. On account of this it is quite difficult to find a sense fiber that runs into the aperture. Nevertheless, when many sections are critically studied, it is possible to see several such connections (fig. 16, A, I-K). Sometimes the slit is shallow (fig. 16, A) and at other times it is deep (fig. 16, C).

As a rule the sense cells are conspicuous. In most cases their sense fibers are broken so that it is generally difficult to trace a sense fiber from the sense cell into the pore, although in these sections almost every pore contains at least a small portion of the sense fiber (*SF*, fig. 16, C, D, H-J). As compared with the hypodermal cells (figs. 17 and 19) the sense cells are large and in most cases lie parallel or oblique to the inner side of the hypodermis. Since most of them lie rather distant from the pores, their sense fibers (fig. 19, *SF*) are long. Their nerve fibers (fig. 19, *NF*) also are long. The size of these cells is not necessarily determined by the size of the pores, because in the legs the pores (fig. 16, A and D) are larger than those (fig. 16, I and J) in the wings where the largest sense cells (fig. 16, E-G) are found.

Many sections, each 6 microns thick, are required to contain all the pores found in group 1 on the front wing. These sections (fig. 19) show that groups 1 and 2 do not lie in the same plane as they appear to lie when observed externally. On the contrary, they lie in two different planes that are almost perpendicular to each other. In other words, the subcosta (fig. 3, *SC*) near its distal end bends at right angles and the radius (fig. 19, *R*) is connected with the subcosta only on the dorsal side. The great size of group 1 is also shown by the fact that most of its sense cells (fig. 19, *SC*) are cut transversely while only a few are seen in a longitudinal view. Five sections, each 10 microns thick, include all the sense cells belonging to group 6 on the femur of the third leg. Thus this group is about 50 microns wide.

In these five sections of a 19-day-old pupa 22 pores and 17 large sense cells (fig. 17, *SC*), besides several smaller ones (*SCNuc'*) were counted.

In the leg of a 19-day-old pupa the hypodermis (fig. 17, *Hyp*) is never any thicker beneath the pores than elsewhere in the

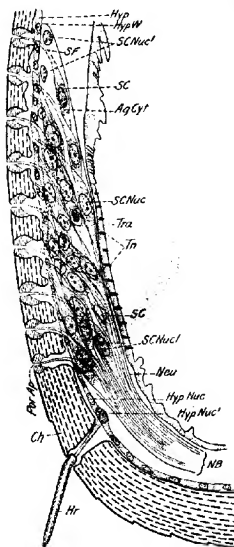


Fig. 17 Combination drawing reconstructed from five consecutive cross sections through femur of third leg of a worker pupa 19 days old. The pores and chitin are drawn diagrammatically and the hair (*Hr*) with its mother cell (*HypNuc'*) were taken from a similar section of a 17-day-old pupa. The other parts were drawn with the aid of a camera lucida. This shows the internal anatomy of group 6. *Hyp*, hypodermis; *HypNuc*, hypodermal nucleus; *HypW*, inner hypodermal wall or remains of basal membrane; *NB*, nerve branch; *Neu*, neurilemma; *Tn*, tænidia of trachea; *Tra*, trachea.  $\times 465$ .

leg, and even at this age it is thin and sometimes nearly wanting. Its small nuclei (fig. 17, *HypNuc*) are widely separated and the hypodermal cell walls (fig. 19, *HypCW*) are never distinct, but appear as an indefinite mass of minute lines, which are represented in most of the drawings by dots. In the legs of more

advanced stages the hypodermis is still thinner and seems to be entirely absent at places. In the legs of younger pupae it is much thicker. Beneath the pores in the wings the hypodermis is much thinner than elsewhere (fig. 19, *Hyp*). In none of these sections older than the 16-day-old pupae was it possible to distinguish the true basal membrane of the hypodermis, although it is very probable that the inner hypodermal wall (fig. 17, *HypW*) is the remains of this membrane. In younger stages of the pupae this membrane is present.

In the 16-day-old worker pupae neither pores nor sense cells are found. At this age many large and deeply staining nuclei occur in the hypodermis where the sense cells later appear. All stages in the formation of hairs may be found. The muscles are not yet completely differentiated and appear as more or less indefinite masses.

In all the sections of legs from the 17-day-old worker pupae, pores are always found, and sense cells are usually present. The chitin and most of the hairs are now fully developed. The hypodermis is much thinner than in the 16-day-old pupae, and in fact most of the various structures are fully differentiated. Thus it is evident that between the sixteenth and seventeenth day there is a rapid change in the various structures and that at this time these sense organs make their appearance. Very often in this and later stages the inner hypodermal wall (fig. 19, *HypCW*) is absent beneath the pores. In such cases it is sometimes impossible to distinguish hypodermal nuclei from sense cell nuclei. The sense cells, therefore, are derived from the hypodermis and are nothing less than modified hypodermal cells that have assumed a different function. In regard to the formation of the pores and cones nothing can be stated because this phase of the subject was not studied.

In sections from the more advanced pupae and in those from the adult bees, pores and sense cells are always present. Very often in sections of legs from pupae 17 days old a large, dark nucleus (fig. 19, *HypNuc*) is present beneath a hair. At first it is easily mistaken for a sense cell belonging to the hair, but a careful investigation always shows that it is the hair mother cell

that has formed the hair. No hairs with sense cells are found in any of these sections.

In the front wing of a 19-day-old pupa the sense cells (fig. 19, *SC*) belonging to groups 1 and 2 occupy only about one-half of the internal space. This leaves a large cavity, the blood sinus (*BlSin*) for the blood, blood corpuscles (*BlCor*), and small tracheal branch (*Tra*). Here as elsewhere the sense cells are surrounded by a membrane, the neurilemma (*Neu*), which appears as a single line. Often its nuclei (*NeuNuc*) are discernible. In the front wing of the adult bee the sense cells (fig. 20) belonging to groups 1 and 2 occupy nearly all of the internal space and leave only a small cavity for the tracheal branch and blood. In the hind wing of both the pupa and adult the space is relatively larger. This indicates not that the sense cells of the adult have increased in number, but in size. Since all the chitinous structures are completely formed by the time the bee emerges from its cell, the same number of pores would be expected in bees just emerged as are found in adult ones. This was ascertained to be the case, as was stated in discussing the disposition of the pores in the worker.

The nerve fibers of the sense cells run immediately into a nerve branch (figs. 15 and 18, *NB*) where as individual fibers they can not be traced farther. However, a longitudinal view of a nerve shows that it is composed of many parallel fibers. In the leg a branch of the nerve proceeds to each group of sense cells, but single fibers (fig. 18, *NF*) run to the isolated sense cells. No two of these branches are given off at the same time. A double-walled membrane, the neurilemma (fig. 18, *Neu*) surrounds the nerve (*N*) and its branches. The walls of the neurilemma contain many small deeply-staining nuclei (*NeuNuc*). Inside these walls among the nerve fibers there are many larger but less deeply-staining nuclei (figs. 18, 20, and 21). A cross section of the nerve shows a network of minute fibers, among which several small dark nuclei (fig. 18, *NeuNuc*) stand out conspicuously. These nuclei in both the nerve and its branches are probably neuroglia nuclei.

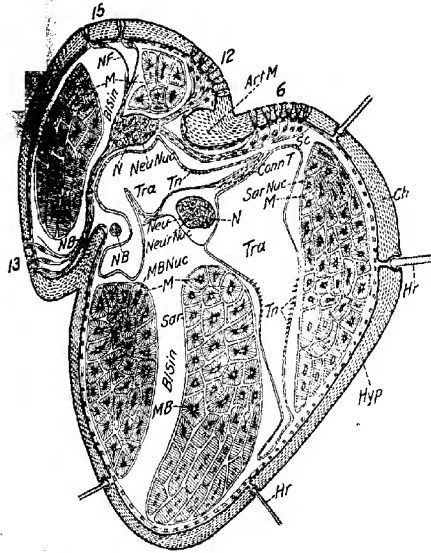


Fig. 18. Semidiagrammatic drawing reconstructed from fourteen consecutive sections through the third leg of a 19-day-old worker pupa. The trochanter and femur were bent at their articulation (*ArtM*) so that they formed an angle of 70 degrees; the sections pass obliquely through this articulation. The smaller portion at the top is the trochanter, only the inner side of which is represented. On this surface are shown groups 12, 13 and 15. The lower portion shows both the inner and outer sides of the femur, group 6 being located on the outer side. The internal parts show the innervation of the olfactory pores and the anatomy of the leg at this place. Cross sections of muscle bundles (*MB*) are shown with lines radiating from the muscle bundle nuclei (*MBNuc*), while longitudinal sections are represented by transverse lines. On account of the bend in the leg the nerve (*N*) is cut transversely in two places.

In the wings of adult bees the nerve is easily traced from the sense cells to the thorax. In one of the sections the innervation is very plain. Here the sense fibers run from the pores to the sense cells from which a nerve (fig. 22, *N*) proceeds all the way to the thorax.

The wing (fig. 22) arises at the bottom of a comparatively large niche in the side of the thorax, then for a short distance it

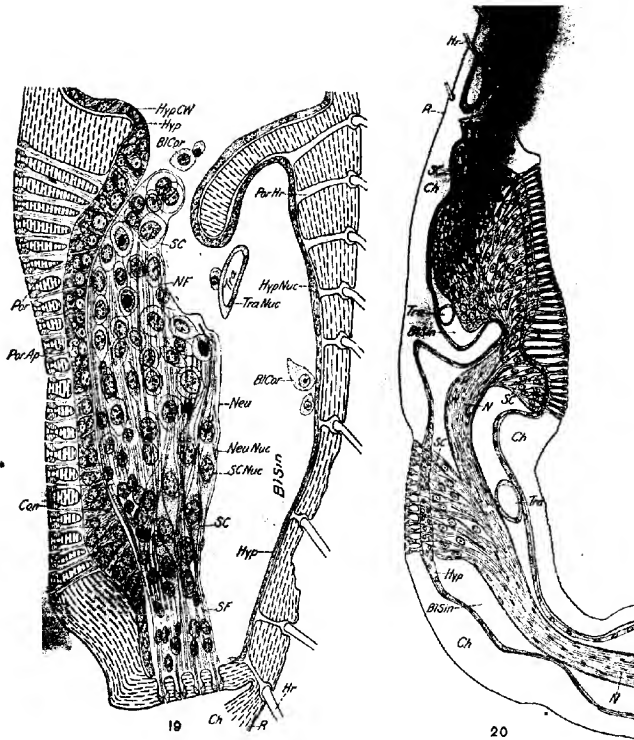


Fig. 19 Cross section (6 microns thick) through the base of a front wing of a 19-day-old worker pupa, showing the olfactory pores and sense cells belonging to groups 1 and 2. The longer row of pores belongs to group 1. All parts were drawn with aid of a camera lucida, except the pores (*Por*), hairs (*Hr*) and hypodermal cell walls (*HypCW*). The correct number of pores is represented; the hairs are drawn as they appear in another similar section, and the hypodermal cell walls are represented only by fine lines.  $\times 465$ .

Fig. 20 Semidiagrammatic drawing from two sections showing the innervation of the three groups of pores on the front wing of an adult worker bee. The longest row of pores is from group 1 and the shortest row is from group 2. The portion showing groups 1 and 2 is from the left front wing and the portion showing group 3 is from the right front wing of the same imago worker.  $\times 185$ .

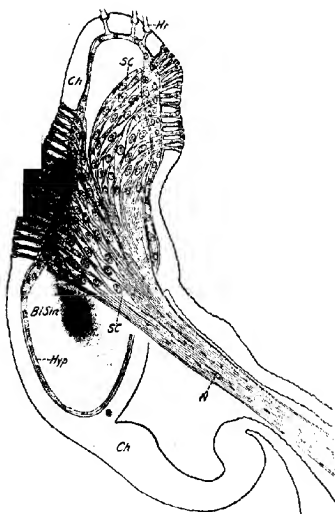


Fig. 21 Semidiagrammatic drawing from two consecutive sections of the hind wing of an adult worker bee, showing the innervation of groups 4 and 5. The longer row of pores is from group 5. The two groups of sense cells (SC) are drawn only approximately in their proper places, for they actually lie slightly one above the other; the trachea (Tra) is also shown slightly out of its normal position.  $\times 185$ .

runs parallel with the side of the thorax to the top of the niche, where it turns at tight angles away from the body. Since the muscles that move the wing are attached only to the sides of the thorax at the lowest part of the wing, muscular tissue is never seen in sections of the wings. The large tracheal branch (fig. 22, Tra) in the wing is also easily traced from the thorax to the sense cells. Although a short distance from the thorax it is cut obliquely into small pieces. Its small branches near the sense cell groups are usually cut transversely but sometimes obliquely. These branches do not follow a straight path and do not extend farther into the wing than the sense cells. Tracheal branches do not persist in any of the veins or even between the articular



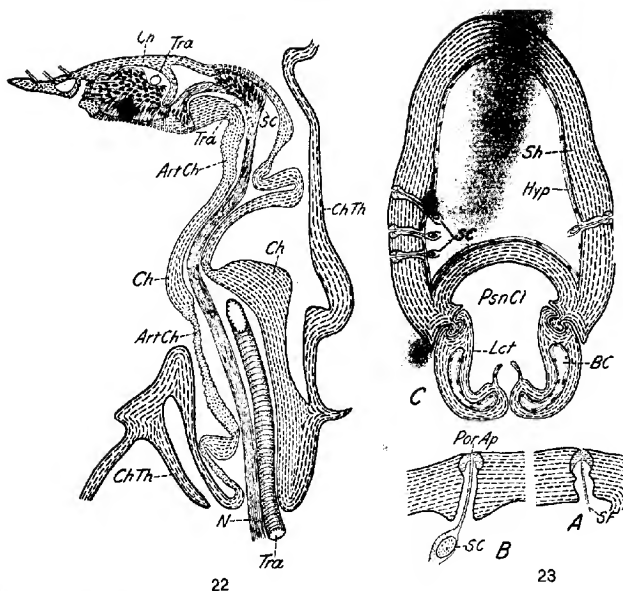


Fig. 22 Semidiagrammatic drawing from a single section of the same imago worker as in figures 20 and 21. This represents the complete innervation of the base of the front wing. The hard chitin (*Ch*) of the wing is shaded with small broken lines, the articular chitin (*ArtCh*) with dots, and the hard chitin of the thorax (*ChTh*) with heavy broken lines.  $\times 78$ .

Fig. 23 Olfactory pores and sense cells (*SC*) seen in cross sections of the sting of a 19-day-old worker pupa. A, pore and its sense fiber (*SF*) from shaft at *e*,  $\times 700$ . B, pore and sense cell from shaft midway between *c* and *e* (fig. 14 A),  $\times 700$ . C, cross section through the shaft (*Sh*) and lancets (*Lct*) of a sting, showing the olfactory pores and their sense cells,  $\times 350$ . In C, the pores, sense cells, and hypodermis are drawn diagrammatically. The remaining parts of C and all of A and B were drawn with aid of a camera lucida.

chitinous layers, whether nearly or completely developed, except where they are associated with the sense cells.

The large nerve of the leg is also easily traced to the thorax. By using several of the sections from the thorax of an adult worker these nerves were followed to large ganglia in the thorax. According to Snodgrass ('10) the prothoracic ganglion innervates the prothorax and the first pair of legs. The second thoracic

ganglion which is a combination of the mesothoracic, metathoracic, and the first two abdominal ganglia, innervates the second and third pairs of legs, the bases of both pairs of wings, the mesothorax, metathorax, the propodeum, and the first abdominal segment.

The groups of sense cells are so located that they are constantly bathed in the blood. The large trachea (figs. 15 and 18, *Tra*) branches profusely, and one of its branches is always near a group of sense cells. There seems to be little connective tissue (fig. 18, *ConnT*) in the leg of a bee. It appears as fine lines and is found between the tracheal branches and nerves. The large muscles (figs. 15 and 18, *M*) are so located that they do not interfere with the sense cells while contracting and relaxing. Hence the sense cells are so located that they are constantly nourished by the blood that surrounds them and they receive their supply of air from the neighboring tracheal branches. Also, the muscles are distant enough not to affect them mechanically.

At various times while examining the superficial structure of the pores in group 3 on the front wing, peculiar structures which were first thought to be hair sockets were noticed irregularly scattered on the tegulae (figs. 2 and 6, *Tg*). Cross sections through these apparent hair sockets (fig. 16, *L*) show that they have the same structure as the olfactory pores. They were observed in only a few sections and their sense cells were never seen. On account of the small size of these structures and since their apertures are never distinct, they were at first not considered as olfactory pores, but since they are identical in structure with the olfactory pores, even if their sense cells were not seen, it is evident that these structures are the external ends of olfactory pores.

Cross sections of stings were made in the same manner as those of the legs and wings, although the slides fail to reveal any sections passing through the extreme end of the lancets.

Since the superficial appearance of the pores in groups 20 and 21 on the lancet (fig. 14, A, F, and G) is identical with that of the pores on the shaft, it may be assumed that the internal anatomy of the pores on the lancet is identical with that of the pores on the

shaft. From the drawings (figs. 16 and 23) it is evident that the structure of the pores on the sting is identical with that of the pores on the legs and wings, and that the pores are opening internally into the body cavity (*BC*) and not into the poison canal of the sting (*PsnCl*).

In regard to the pores of the sting Snodgrass (1917) says:

The reader will see often repeated the statement that the poison leaves the sting both by a ventral opening between the lancets near their tips and by several lateral pores near the ends of the lancets opening from the poison canal upon the bases of the barbs. The writer, however, has never been able to observe the exit of the poison from any such lateral pores, while, on the other hand, it is very easy to watch it exude from between the lancets on the ventral side of the sting near the tip.

An examination of the end of each lancet does reveal a number of oblique pores which have been figured by other writers, and they certainly open on the bases of the barbs as described, but their inner ends apparently communicate with the body cavity (*bc*) of the lancet instead of passing clear through the lancet and opening into the poison canal. Furthermore, a paired series of exactly similar pores extends the entire length of the shaft of the sheath, opening on its dorsal surface from the body cavity. No one could possibly claim that the poison emerges also through these pores, which, very curiously, do not appear to have been described before, although they are even more conspicuous as well as more numerous than those of the lancets. The writer has not been successful in preparing histological sections of the sting which show these pores, but they probably constitute the ducts of some kind of subcuticular glands.

From the foregoing quotation it is clear that Snodgrass considers these pores as communicating with the body cavity of the shaft and lancet and not of the poison canal. But instead of being some kind of a subcuticular gland, they are connected with sense cells and are thus sense organs.

#### Summary

To sum up, then, the olfactory pores consist of inverted flasks in the chitin and spindlelike sense cells lying beneath the mouths of the flasks. The bottom of the flask forms an external covering and about two-thirds of the space at the bottom of the flask is occupied by a hollow chitinous cone which is not separated from the surrounding chitin, but only stains less deeply.

The sense organ of the sense cell pierces the bottom of this cone and enters a round, oblong, or slitlike external chitinous pore aperture. The morphology of the pores and the sense cells in the wings, legs, and stings is identical, with the exception of variations in size and shape. The complete innervation for those in the wings and legs is also identical. The interior of the base of the cone is literally a solid mass of sense cells while in the legs and stings these cells occupy comparatively little space. In the wings the pores and sense cells are first noticed in the 17-day-old pupa, and the sense cells do not reach their full size until the bee arrives at the adult stage, although all the pores seem to be present in the 19-day-old pupa.

Judging from the structure of these organs it is observed that the cytoplasm in the end of the sense fiber just beneath the pore aperture is constantly in contact with the external air. In speculating as to the function of such an apparatus, all mechanical senses, such as sight, touch, and hearing, must be excluded, and these organs may be presumed to react to some kind of a chemical stimulus, as humidity, temperature, taste, or smell. Without hesitation taste may be excluded, for the food of a bee never comes in contact with the parts containing these pores.

#### PHYSIOLOGY OF OLFACTORY PORES

To ascertain whether the pores which have been studied are actually the organs in the bee that receive odor stimuli, the wings, legs, and stings of many workers were operated upon. The behavior of these individuals was carefully studied and they were tested with various odors as described on page 280.

##### *Wings cut off*

All four wings of 50 middle-aged workers were cut off close to the thorax with small dissecting scissors. An examination of these wings showed that only occasionally did the scissors pass through a group of these pores. These bees were entirely normal, judging by their behavior and longevity. They lived from 18 hours to 16 days, with 9 days and 23 hours as an average.

When tested with odors they responded normally but slightly more slowly. The average reaction time to oil of peppermint was 2.4 seconds, to oil of thyme 3.3 seconds, to oil of wintergreen 3.5 seconds, and the average 3 seconds. This is 0.3 second slower than the general average of unmutilated bees with the same odors. The workers tested were 17.

#### *Wings pulled off*

All four wings of 150 middle-aged workers were pulled off. This is accomplished by holding the bee firmly on a table and by giving each wing a quick jerk, severing it at its articulation. A microscopical examination of the detached wings showed that they possessed all the groups of pores. Judging by their behavior and longevity these wingless workers were otherwise normal in every way. When first introduced into observation cases, they immediately busied themselves in cleaning each other, during which operation they often licked up the drop of yellowish blood that exuded where the wing was detached. They also ate freely, fed each other, never fought, and, in fact, behaved as normally as unmutilated bees in such surroundings. They lived from 3 hours to 17 days, with 9 days and 20 hours as an average.

Strange bees which were put among them were at once recognized as strangers. The wingless ones ran after the strangers, bit their legs and pulled their wings, but only occasionally did a wingless bee seize a stranger, and not once did one attempt to sting a strange worker. This treatment lasted only a few minutes, for in a short time the wingless bees fed, cleaned, and even stroked the strangers with their antennae. The behavior toward strange bees was entirely different than that shown by those with their wings cut off just beyond these pores. Pulling off the wings later proved to be a good precaution before mixing strange bees. It is slow but always effective, because they never kill one another even though they may be slightly hostile at first.

These wingless bees were tested with odors in the usual manner. Their responses, when they reacted, were similar to those

of uninjured bees, but always much slower. They often failed to respond at all, although they were otherwise as active as unmutated bees. A failure to respond within 60 seconds after an odor was held under them was regarded as entirely negative for that odor. The most general responses were to move away slowly and to vibrate and stroke the antennae. The average reaction time to oil of peppermint was 18.8 seconds, to oil of thyme 20.6 seconds, to oil of wintergreen 27.3 seconds, to honey and comb 28.8 seconds, to pollen 38.5 seconds, and to leaves and stems of pennyroyal 28.8 seconds. The average was 27.1 seconds. Twenty-eight workers were tested. For unmutated bees with the same odors the general average reaction time was 3.4 seconds; therefore by removing the pores on all four wings the average reaction time is increased eight times.

*Bases of wings glued*

For fear that the bees with their wings pulled off were abnormal in some unknown way, the bases of all four wings of many workers were glued by applying a small amount of liquid glue to each side of the wing near the articulation. When the glue was thoroughly dry these workers were put into observation cages. The glue caused the wings to be stiff so that they could not be vibrated, but judging from their behavior and longevity the bees were normal in all other respects. They lived as long as unmutated bees, and when strange workers were put among them they were always accepted without much hostility. The responses were the same as for the wingless bees. The average reaction time to oil of peppermint was 12.5 seconds, to oil of thyme 15.6 seconds, to oil of wintergreen 27.5 seconds, to honey and comb 38.8 seconds, to pollen 36.8 seconds, and to leaves and stems of pennyroyal 38.8 seconds. The average was 28.2 seconds. Twenty workers were tested. Thus by covering all the pores on the wings with glue the reaction is increased eight times. That the odor of this glue does not affect the results is shown on page 296.

*Pores on legs covered*

The entire trochanter, the proximal end of the femur, and the tibia of all six legs of 36 workers were covered with a thin layer of vaseline and beeswax applied with a small paintbrush. The proper stiffness of the mixture was obtained by mixing three parts of melted yellow commercial vaseline with one part of melted beeswax. Great precaution was taken not to apply too much of this mixture, because when a bee so treated attempted it invariably begins at once to clean itself and often rubs the mixture on the spiracles and dies quickly. After a short time these workers ceased to clean themselves, began to eat, and were quite normal in their behavior. On an average they lived as long as untreated bees and succeeded in cleaning off all the mixture by the fourth day. They were tested two or three hours after being treated and responded normally but slightly more slowly. The average reaction time to oil of peppermint was 4.2 seconds, to oil of thyme 6.2 seconds, to oil of wintergreen 5.1 seconds, to honey and comb 18.8 seconds, to pollen 9.7 seconds, to leaves and stems of pennyroyal 11 seconds. The average was 8 seconds, or two and one-half times the average for untreated bees with the same odors. Twenty workers were tested.

Since this mixture of vaseline and beeswax gives off a slight odor, there was a possibility that the odor itself caused the foregoing differences in reaction time. To ascertain this, a still larger amount of this mixture was placed on the antero-dorsal side of the abdomen, and the bees were tested with various odors. In such a position the bee can not reach it and therefore can not escape the odor of the mixture. The average reaction time to oil of peppermint was 2.5 seconds, to oil of thyme 2.6 seconds, to oil of wintergreen 3.1 seconds, and average 2.73 seconds. Eighteen workers were tested. The same odors with normal bees gave an average reaction time of 2.64 seconds. The odor of the mixture, therefore, did not affect the reactions to other odors.

*pulled off and pores on legs covered*

For this experiment 33 of the 150 wingless bees mentioned on page 334 were used. The pores on the legs were covered with the mixture of vaseline and beeswax four days after the wings had been pulled off. When put into observation cages, these workers were quite hostile to each other, they bit and pulled one another's legs and wings for at least three hours, after which they cleaned and behaved as usual. They were otherwise normal in all respects and lived on an average 9 days and 5 hours. When strange bees were put among them, they were quickly attacked and were soon killed. In reacting to odors their behavior was similar to that of un mutilated bees, but much slower. The reaction was at times entirely negative to all the six odors. The average reaction time to oil of peppermint was 27.3 seconds, to oil of thyme 44.1 seconds, to oil of wintergreen 39.3 seconds, to honey and comb 41.7 seconds, to pollen 44.5 seconds, and to leaves and stems of pennyroyal 42.9 seconds, and the average of all was 40 seconds. The workers tested numbered 20. By removing all the pores on the wings and by coating most of them on the legs the time of response to the six odors was increased 12 times.

The experiment just described may be criticised on the ground that the removal of the wings of bees causes an abnormal condition which is brought about by the injury of the operation, and that the reaction time is affected on account of the injury. When the behavior of bees thus mutilated and their longevity are considered it will be clear that the injury itself does not cause the slow responses. It may also be urged that any substance which is used to cover the pores comes in contact with the peripheral ends of the sense fibers and this contact might make the insect abnormal, so that even if these organs were not olfactory, the bee would not respond to odor stimuli. Such a contact is scarcely possible for two reasons: (1) Since the parts of the bee where the pores are located are covered with a dense coat of hair, the liquid glue or mixture of vaseline and beeswax probably



does not come in direct contact with the chitin and the pores; (2) as the sense fibers end in the neck of the funnel-like aperture and do not extend into the mouth of the funnel, their peripheral ends do not attain the level of the external surface of the chitin. It thus seems impossible for these thick substances to run into the mouths of the funnel-like apertures, where they must force out the air in order to come in contact with the ends of the sense fibers.

To anesthetize the ends of the sense fibers with a finely powdered anesthetic, either by blowing or by applying the powder with a brush on the parts possessing the pores, is impossible for two reasons: (1) The powder does not come in contact with the chitin on account of the dense coat of hair; (2) the diameter of the smallest particles of the powder is microscopically two or three times that of the largest pore apertures.

To anesthetize the ends of the sense fibers by using an anesthetic dissolved in water, the following procedure was employed: With a small pencil brush the pores on the legs and wings were covered with a one-fifth saturated solution of alypin, a synthetic anesthetic. The bee was immediately put into a small observation case and the latter was held in the current of an electric fan for five minutes. By this time the bee was again dry and appeared normal in most respects, but it was extremely restless and did not eat candy until several minutes afterwards. Since the bee did not become perfectly quiet until two or three hours afterwards, a reaction time in seconds could not be obtained while the anesthetization was effective. In view of the fact that the rate of walking of un mutilated bees may be increased by using odors, this anesthetized bee was tested with oil of peppermint to determine whether odors cause its rate of movement to be increased. The word excited is used to describe the action of the bee when it quickly moved from over the odor and vigorously vibrated its wings. In all, 10 workers were used, with the following results:

Two workers: During the first 10 minutes their slow rate of walking was not increased; after 15 minutes one was easily excited; after 30 minutes the other could not be excited and in-

creased its rate only slightly, and after 40 minutes it moved a little faster when tested.

One worker: During the first 3 minutes it did not increase its rate, but later was easily excited.

Five workers: During the first 5 minutes they increased their rate only slightly, but later were easily excited.

Two workers: At first, as well as later, they were easily excited.

A control was used by applying water in the same manner as the anesthetic, with another pencil brush. In all, 10 workers were used, and each one without failure was easily excited at first as well as later. It may be suggested that only a relatively small amount of any liquid is able to reach the ends of the sense fibers, for two reasons: (1) On account of the dense coat of hairs, and (2) because the liquid must replace the air in the mouths of the funnel-like apertures.

#### *Stings extracted*

For this experiment 20 of the 100 bees with their stings extracted, as mentioned on page 278, were used. In most respects these bees appeared normal, but they lived only 30 hours on an average. They responded to odors normally. The average reaction time to oil of peppermint was 2.6 seconds, to oil of thyme 3 seconds, and to oil of wintergreen 3 seconds. The average was 2.8 seconds. This is 0.2 second more than the average for the same odors when un mutilated bees were used.

#### SUMMARY

Bees with their antennae mutilated in the slightest degree, either by amputation or by varnishing them, are never absolutely normal. The more joints of the antennae severed, the more abnormal the bees are. There is nothing in the results of these experiments to indicate that the antennae play any part in olfaction. The maxillae, labial palpi, proboscides, mandibles, and stings may receive odor stimuli in a limited degree but this is not certain. Neither the mouth cavity nor the epipharynx plays

any part in receiving odor stimuli. When the wings are cut off distal to the olfactory pores, the reaction times are not materially changed. When the wings are pulled off and the bases are glued the reaction time is increased eight times. Sister bees so treated are not hostile to each other and do not actively fight strange bees. Such facts indicate that the olfactory organs are sufficiently destroyed or prevented from functioning by these manipulations so that the bees do not readily recognize enemies. With the pores on the legs covered, the reaction time is increased two and one-half times. When the wings are pulled off and the pores on the legs are covered, the reaction time is increased twelve times. Sister bees so treated are slightly hostile to one another and quickly attack strangers. After fighting a short time they distinguish friends from enemies, cease attacking their friends, but never cease fighting their enemies until the strangers are killed. Neither the odor of glue nor of vaseline affected these results.

TABLE 2

*Summary of experiments with worker bees to determine the location of the olfactory organs*

EXPERIMENT	AVERAGE REACTION TIME		NUMBER OF WORKERS TESTED	AVERAGE LENGTH OF LIFE IN THE OBSERVATION CASE	
	For three odors	For six odors		days	hours
Unmutilated.....	2.64	3.4	37	9	3
Glue on thorax as control.....	2.76		19	9	3
Vaseline on abdomen as control.....	2.73		18	9	3
Flagella burnt off.....	4.0		7	0	17
Flagella glued.....	2.9		21	1	0
Maxillae and labial palpi cut off.....	3.3	4.0	19	1	0
Proboscis cut off.....	2.9		22	0	7
Mandibles cut off.....	3.5	4.8	20	7	0
Flour paste in mouth.....	2.68		20	7	12
Wings cut off beyond pores.....	3.0		17	9	23
Stings extracted.....	2.86		20	1	6
Wings pulled off.....	22.2	27.1	28	9	20
Bases of wings glued.....	18.5	28.2	20	9	3
Pores on legs covered with vaseline.....	5.2	8.0	20	9	3
Wings pulled off and pores on legs covered with vaseline.....	36.9	40.0	20	0	5

As the structure of these pores is suitable for receiving chemical stimuli, since all the foregoing experiments with these organs indicated olfactory function for them, it may now be assumed that these pores constitute the olfactory organs in the honey bee, and perhaps in insects in general.

Table 2 gives the results of all experiments with worker bees in timing their responses to various odors. The "three odors" used are those from oil of peppermint, oil of thyme, and oil of wintergreen. The "six odors" used are those from oil of peppermint, oil of thyme, oil of wintergreen, honey and comb. pollen, and leaves and stems of pennyroyal.

#### GENERAL SUMMARY AND DISCUSSION

The behavior of bees in observation cases is very similar in many respects to that in the hives. Bees have a very acute sense of smell. This sense is most highly developed in the drones and least developed in the queen while that of the worker is scarcely inferior to that of the drone. Olfactory pores are found on the bases of all four wings, widely scattered on the trochanter and at the proximal ends of the femur and tibia of all six legs, on the second and third tarsal joints of most legs and generally distributed on the shaft and lancets of the sting. Each pore is a chitinous structure connected with a bipolar sense cell, the peripheral end of which comes into direct contact with the external air. Such sense cells are met with in all insects for Künchel and Gazagnaire ('81) assert that bipolar sense cells are common to all insects.

Bees with their antennae mutilated in the slightest degree, either by amputation or by covering the surface, are never absolutely normal. The more joints of the antennae severed, the more abnormal the bees are. The antennae probably play no part in receiving odor stimuli. The maxillae, labial palpi, mandibles, and sting either receive odor stimuli to a limited degree or the mutilation in the experiments on these parts caused a slight difference in the reaction time. Judging from their behavior and longevity, bees with the wings either pulled off or glued at the base and with the pores on the legs covered are normal, except

that their ability to smell is greatly reduced. In the honey bee these pores are olfactory organs, and perhaps the same is true for all insects because they are common to all orders of insects and possibly are homologous to the lyriform organs of spiders (McIndoo '11). As yet they have never been looked for in crustaceans, but it is very probable that this class of arthropods possesses similar organs.

The following criticisms concerning the physiological experiments performed with the antennae of various insects may be offered. Most of the previous observers have studied the behavior of the insects investigated in captivity for only a short time, while the remainder have paid no attention at all to the behavior of their un mutilated insects. They cut off either a few joints of both antennae, or these entire appendages, or varnished them with paraffin, rubber, and so forth. When a few joints are severed the sense of smell is apparently weakened. This is true for bees also. When both antennae are amputated or varnished the insects, as a rule, fail to respond to substances which normally affect the olfactory sense. They generally fail to respond to odors held near them and fail to find food in captivity, and do not return to putrid meat and dead bodies when removed from such food. Males so mutilated as a rule do not seek females and show no responses when females are placed near them. Such experiments were seriously criticised until Hauser in 1880 presented his apparently conclusive results. Many of the insects on which he experimented with the antennae amputated became sick and soon died. Most of them failed to respond when the antennae were mutilated, although *Carabus*, *Melolontha*, and *Silpha* responded slightly, while all the Hemiptera that he used responded almost as well with their antennae off as they did with them intact. Only 40 per cent of the ants from which Miss Fielde ('01) cut the antennae recovered from the effect of the shock. Not one of these observers has studied the behavior of the species under observation sufficiently to know exactly how long they live in captivity with their antennae either intact or mutilated. No one, except Miss Fielde, has kept a record of the death of the mutilated and normal insects

so accurate that one might know what percentage died from the operation. To cut off some other appendage or even the lower part of the head, as Forel ('74, '85) did, is not a fair test, because such operations seldom expose sense cells and never any nerve equal in size to that of the antenna, unless one pulls off the wings. When the wings are pulled off the large nerve is severed between the masses of sense cells and thorax, and the sense cells are not exposed to the air, as they are when antennae are removed. Even if the antennae are cut through the scape, the large masses of sense cells belonging to Johnston's organs (Child '94) are severed. When the lower part of the head or the tarsi are cut off, as Forel did, no nerves are exposed to the air except ends of small nerves. From the foregoing it is only reasonable to assume that when the antennae of any insect are injured in the least degree, the insect is no longer normal and if it fails to respond to odors placed near it, this negative response may be due to the shock of the injury.

The following criticisms based on a consideration of the morphology of the antennae may also be offered. In the honey bee the pore plates (Schenk '03) can scarcely be considered as olfactory organs, because the drone has almost eight times as many as the queen, and responds to the odors presented in slightly more than one-half the time. It is true that those of the queen are considerably larger, but even on this basis the reaction times are not comparable. The pegs may be entirely eliminated as olfactory organs, because they are absent in the drone, but are abundant in the worker and the queen. Drones, queens, and workers have about the same number of Forel's flasks and pit pegs. Schenk's view that the pegs receive odor stimuli in the queens and workers, while Forel's flasks and the pit pegs function in this way in the drones is inconsistent, because if the latter two structures function for such a purpose in the drones why should they not also in the females? Since these two structures are few in number and many times smaller than the pegs, we can not compare them physiologically. Thus it is seen that not one of these antennal organs of the honey bee offers a solution for the ratios obtained with the use of the various odors.

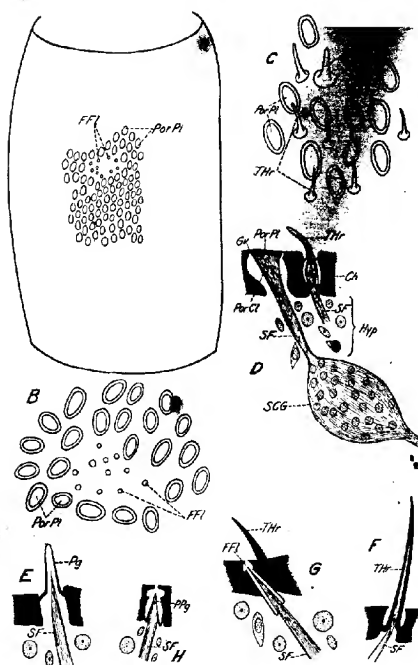


Fig. 24 Antennal organs of the honey bee copied from Schenk. A, an antennal joint of a drone, showing a few of the many pore plates (*PorPl*) and a group of Forel's flasks (*FFI*),  $\times 100$ ; B, pore plates and Forel's flasks from a drone's antenna,  $\times 400$ ; C, pore plates (*PorPl*), pegs (*Pg*), and tactile hairs (*THr*) from a worker's antenna,  $\times 400$ ; D, internal anatomy of a pore plate and of a tactile hair; E, the same of a peg, F, the same of a tactile hair; G, the same of a Forel's flask; H, the internal anatomy of a pit peg. D - H,  $\times 500$ .

If the reaction time of each caste is compared with the total number of olfactory pores a consistent inverse ratio is obtained. A drone has 2600 pores and responds in 2.9 seconds; a worker possesses 2200 pores and responds in 3.4 seconds and a queen has 1800 pores and responds in 4.9 seconds.

Pore plates are not the olfactory apparatus in all insects, because they are entirely absent in the *Lepidoptera*. The pegs

can not be the olfactory organs in all insects, for they are absent in many male bees and almost wanting in Lepidoptera, although possibly the end rods in butterflies and moths are homologous. According to Vom Rath ('95) pegs are found not only on the antennae and mouth parts but also all over the body, and Nagel ('94) found them elsewhere than on the antennae. If the pegs are the olfactory organs and if insects with amputated antennae are normal, then why do not such insects respond positively at least slightly to odors instead of negatively, as most observers claim?

It is certain that spiders can smell, yet they have no antennae nor any organs that may be compared to the antennal organs of insects. Hence, this is another argument against the antennae being organs of smell. All insects either have antennal organs like those described for the bee, or modifications of them, yet no two authors who have studied them have agreed concerning their function. Such chaos can be replaced by facts, only when the behavior of the insects investigated is thoroughly studied and when experiments are performed in ways other than on the antennae alone. Then it will be realized that the antennae can no longer be regarded as the seat of the sense of smell in insects.

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TRANSMISSION THROUGH THE RESTING EGG OF  
EXPERIMENTALLY INDUCED CHARACTERS  
IN *ASPLANCHNA AMPHORA*<sup>1</sup>

CLAUDE W. MITCHELL AND J. H. POWERS

The chief purpose of undertaking the experiments which form the basis of this article was to ascertain whether or not the marked modifications which may be experimentally induced in the above rotifer are in any sense hereditary beyond the parthenogenetic stages. As to what the modifications are in this remarkably variable and tri-morphic species and as to the means by which they are produced in nature as well as under experiment, we refer the reader to the previous articles in the present series, as well as to the publications of Lange, Powers, and Daday (see bibliography). We may state briefly that variation in this species is shown to be, in last resort, entirely under the control of nutrition; that in character it is deep-seated, pervasive, erratic, and sudden—bearing all the ear-marks of mutation; and that it is hereditary in a strong though variable degree throughout the parthenogenetic series.

In several respects variation in this rotifer appears like a species-making process: The different types produced occur erratically though sometimes abundantly in nature; their differences would be quite sufficient for specific distinction, and several of the forms produced closely parallel types of the genus which have been assumed to be definite species. Nevertheless it is plain that we can not regard the variation of this *Asplanchna* as a true species-forming process until it is proven that the variation in question effects, in at least some degree, the sexual gametes. However varied the parthenogenetic forms and however thoroughly it may be proven that this variation is a result of a modification of the parthenogenetic ova, which transmits itself for

<sup>1</sup> Studies from the Zoological Laboratory, The University of Nebraska, No. 110.

generation after generation, it is clear that nothing akin to a new species is formed if sexual reproduction again brings the species back to its original form.

This seems at first sight to be the case. All the fertilized or resting eggs hatch as individuals of the small saccate type, which represents in general the primitive phylogenetic form of the entire genus. The astonishing variations of the parthenogenetic series, germinal though they are, thus seem to be wiped out by sexual reproduction much as the somatic variations of the complex metazoan are wiped out by the same process. The question which we wish to answer is thus, whether this wiping out process is complete; whether the fertilized egg is so different a thing from the parthenogenetic egg; whether the entire variation of the species, and doubtless of the genus as a whole, is but a play of the environment upon parthenogenesis as such, or whether it is the result of the forces fundamentally modifying the gametic constitution of the species.

It should be pointed out that the degree of inheritance through the resting or fertilized egg must obviously be less than the usual degree of inheritance through the parthenogenetic egg, since the latter shows at least a strong tendency to maintain the species or at least the race in question in a constant physiological and morphological state. This the resting egg does not do; the first generation of young hatching from it, is always *morphologically* the same, from whatever type of the species the egg is derived. There is still, however, the possibility that the young saccate individuals derived from different eggs differ *physiologically* and in their reproductive tendencies.

Evidence has accumulated during our work on this rotifer which tends to show that a parthenogenetic series of these small animals, metazoa though they are, conducts itself very much as does a series of protozoa derived from a single conjugant. Such a series may thus be compared, with not a little truth, to the somatic development of a larger metazoan resulting from the fertilized egg. Now, just as in the metazoan, characters may crop out at late stages and after a multitude of cell generations, which are none the less determined by the inherent qualities of the ferti-

lized egg, so it is readily conceivable that in the parthenogenetic series of rotifers the tendency for certain form-changes to occur in generations more or less removed from the resting egg may none the less have been determined by the nature of that egg as such. In short, there may be hereditary. Or, to put the same thing in a different way for clearness, it is conceivable that inheritance through the resting or fertilized egg of this rotifer need not manifest itself in the visible characters of the individual which emerges from this egg; it may be hidden as tendencies only to manifest itself in later generations.

A few terms may be briefly defined as an aid to readers not familiar with our preceding studies upon *Asplanchna*. A pedigree-series is a succession of individuals derived, by parthenogenetic reproduction, from a single resting or fertilized egg. One of the early members of each generation is usually isolated to become the parent of the succeeding generation. The term metabolic rhythm has been used to indicate an internal rhythm which can be measured by the rise and fall in the number of young born, under normal conditions, to the individuals constituting the generations in the pedigree series. The words, type, form, and stage, refer to the tri-morphic conditions in which this rotifer is found. The word potential is used to indicate the different physiological levels as represented by these three different stages.

#### EXPERIMENTS

All the *Asplanchna* employed in the following experiments were derived from stock used in the previous year in the studies upon sex-determination. A single mass culture had been carried through the summer. Four healthy individuals were chosen from this in the fall and were tested by allowing them to multiply rapidly in a single mass culture. The stock seemed good: In a few days many had been born and even males had been produced. On October 7 a single individual was chosen to become the parent of the mass cultures from which all series and cultures used through the experiments were derived, all other material being entirely discarded.

The initial mass culture thus derived proved remarkably strong. By October 10, but a few days after the isolation of the single progenitor, hundreds of rotifers were present. Males were also first seen on October 10, and the following day individuals containing resting eggs were present.

From this mass culture derived, as we have seen, from a single individual, four series, in two contrasting groups, were started as follows: First, one of the above-mentioned individuals containing resting eggs was isolated on October 11. Its eggs, dropped two or three days later, were placed in watch glasses containing fresh tap water, where they began to hatch by October 24. Two of these young were chosen to become the parents of series *H* and *I*, which, being similarly derived, constitute one group. Other sister eggs hatched and were used to found general mass cultures serving no other purpose than as subsidiary controls.

Second, another group of two series was started in a different way. The general mass culture from which the original progenitor of *H* and *I* had been chosen was fed in a special manner so as greatly to increase its numbers, to induce cannibalism, and thereby produce a number of the third or campanulate type of the species. These campanulates were active cannibals in their own culture before their isolation. After isolation they were fed upon numbers of the saccate form of their own species. Male production soon followed. As soon as this was noted a single young campanulate was isolated and fertilized by a large male produced by another campanulate mother; resting eggs were produced, and after suitable treatment began to hatch on December 2. Two of these young were used to become the direct progenitors of the two series *J* and *K*.

The relation of the above-mentioned four series should thus be clear: All are the descendants of one parthenogenetic female although many generations removed. *H* and *I* are derived from individuals which are full sisters hatched from resting eggs produced by the humped type. *J* and *K* are likewise full sisters but hatched from fertilized eggs derived from the still higher or campanulate stock. Thus the immediate progenitors of these

series, although of common descent, were of different morphological types and of what we have called two different potentials or different physiological states artificially produced from what was at the start an apparently homogeneous parthenogenetic stock. The purpose of the experiment is to see whether the advent of sexual reproduction at the beginning of each of these series will have obliterated these different potentials, or in other words, whether the four series derived in these two different manners will be different or identical when maintained and bred under identical conditions. These four contrasting series were reared as follows: From *H* one hundred and forty-five generations; from *I*, eighty; from *J*, fifty-five; and from *K*, eighty-seven. Table 1 gives the histories of these four series.

Examination of table 1 shows the astonishing difference between the two groups—series *H* and *I* on the one hand, and series *J* and *K* on the other. Equally striking is the parallelism between the two series in each group. *H* and *I* remain alike practically throughout the entire experiment; *J* and *K* equally so. It will be seen that *H* and *I* are all but homogeneous saccate series. They are as constant as were series *D* and *D 2* recorded in the first paper of these studies. Temporary transitions to the humped type do occur, but they are rare and only at times when the metabolic rhythm was at its height. It should be distinctly stated that *H* and *I* were not weak races.

There were no signs of decadence in either series, even though *H* was carried to the one hundred and forty-fifth generation. Both series might have been prolonged had time permitted. The average number of young produced per individual approximated closely to sixteen in *H* and to fifteen in *I*.







TABLE 1 (Continued)

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOUNDED BY GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PERCENT OF ♂	PERCENT OF ♀	TYPE	REMARKS
54	1912 26	16	27	2	0	15	1	0	100	S	
55	28	20	28	3	0	20	0	0	100	S	
56	30	16	30	1	5	11	0	31	69	S	
57	31	9	31	2	3	6	0	33	67	S	
58	Feb. 2	18	Feb. 2	1	6	11	1	32	68	S	
59	3	19	4	2	3	15	1	17	83	S	
60	5	22	5	1	2	20	0	9	91	S	Prod. H
61	7	18	8	4	9	9	0	50	50	S, I	Prod. H
62	10	16	10	1	2	14	0	13	87	S, I	
63	12	5	12	1	0	5	0	0	100	C	Ate Y
64	14	7	14	1	0	5	2	0	100	C	Ate Y
65	17	7	17	1	1	5	1	16	84	C, S	
66	18	8	18	1	0	8	0	0	100	H, S	
67	20	20	20	1	0	19	1	0	100	S	
68	21	20	21	1	4	16	0	20	80	S	
69	23	21	23	1	5	16	0	24	76	S	
70	25	13	25	3	0	11	2	0	100	S	
71	27	6	27	1	0	6	0	0	100	S	
72	29	20	29	1	0	19	1	0	100	S	
73	Mar. 2	10	Mar. 2	1	3	7	0	30	70	S	
74	3	22	3	1	4	18	0	18	82	S	
75	5	19	5	1	4	15	1	22	78	S	
76	7	16	7	1	0	1	1	0	100	S	
77	9	20	9	1	0	19	1	0	100	S	

		Apr.		Apr.		Prod. H.		Disc.		Rest. disc.	
78	11	22	11	1	0	21	1	1	0	100	S
79	12	21	12	1	6	15	0	0	29	71	S
80	14	16	14	1	0	13	1	1	0	100	S
81	16	18	16	1	1	17	0	6	6	94	S
82	17	18	17	1	0	18	0	0	0	100	S
83	18	20	18	1	7	13	0	0	35	65	S, I, H
84	20	21	20	1	6	15	0	0	29	71	S
85	21	21	21	1	2	19	0	9	91	91	S
86	23	22	23	1	2	19	1	9	91	91	S
87	26	14	26	2	0	14	0	0	0	100	S, I
88	28	23	28	4	4	10	0	17	83	83	S
89	29	15	29	1	6	9	0	40	60	60	S
90	30	12	30	4	9	3	0	75	25	25	I, H
91	1	23	1	1	14	7	2	66	33	33	H
92	2	20	2	1	14	6	0	70	30	30	H
93	4	25	5	5	12	8	5	60	40	40	H
94	6	15	6	1	0	15	0	0	100	100	I, H
95	7	19	7	1	1	17	1	6	94	94	1
96	9	17	9	1	1	17	0	0	100	100	S
97	10	10	10	1	8	2	0	80	20	20	I
98	11	22	11	1	10	11	1	47	53	53	I
99	13	22	13	1	6	16	0	27	73	73	H
100	14	18	14	1	0	17	1	0	100	100	I, I
101	16	19	16	1	8	11	0	42	58	58	I, S
102	17	20	17	2	3	17	0	15	85	85	S, I, S
103	19	19	19	1	0	19	0	0	100	100	S
104	21	22	21	1	0	22	0	0	100	100	S
105	22	22	22	1	0	22	0	0	100	100	S
106	24	17	24	1	0	16	1	0	100	100	S



History of Series I										
1911										
Oct.										
1	24	1	28	1	0	18	0	0	100	Rest Disc.
2	26	18	29	1	1	20	0	5	95	Rest Disc.
3	28	21	30	1	0	17	1	1	100	Rest Disc.
4	30	18	31	1	0	17	1	0	100	Rest Disc.
5	1	18	June 1	1	1	2	13	0	88	Rest Disc.
6	2	15	2	1	2	13	0	12	100	Rest Disc.
7	4	24	4	2	0	23	1	0	100	Rest Disc.
8	6	13	6	1	0	12	1	0	100	Rest Disc.
9	8	16	8	2	0	12	4	0	100	Rest Disc.
10	10	12	12	3	0	8	4	0	100	Rest Disc.
11	4	8	15	2	0	8	0	0	100	Rest Disc.
12	18	5	18	1	0	5	0	0	100	Rest Disc.

TABLE 1—Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF FIRST YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIV- IDUALS PARENT IN FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PER CENT OF ♂	PER CENT OF ♀	TYPE	REMARKS
13	1911 Nov. 20	18	Nov. 20	1	0	17	1	0	100	♀	
14	21	11	25	11	0	8	3	0	100	♀	
15	26	3	27	2	0	2	1	0	100	♀	
16	28	10	29	2	0	9	1	0	100	♀	
17	1	14	Dec. 1	1	1	13	0	7	93	♀	
18	2	22	2	1	1	21	0	5	95	♀	
19	4	23	4	1	1	21	1	5	95	♀	Sis. Pro. H.
20	5	20	5	1	0	20	0	0	100	♀	Sis. Pro. H.
21	7	19	7	1	1	18	0	5	95	♀	Sis. Pro. H.
22	8	20	8	2	0	20	0	0	100	♀	
23	9	12	9	1	0	11	1	0	100	♀	
24	10	20	10	1	0	20	0	0	100	♀	
25	12	10	12	1	2	8	0	20	80	♀	
26	14	23	14	1	0	23	0	0	100	♀	
27	15	21	15	1	0	20	1	0	100	♀	
28	16	15	17	3	0	13	2	0	100	♀	
29	18	7	18	1	0	7	0	0	100	♀	
30	20	9	20	1	0	9	0	0	100	♀	
31	22	5	22	1	0	5	0	0	100	♀	
32	24	13	25	5	0	10	3	0	100	♀	
33	27	19	27	1	0	18	1	0	100	♀	
34	29	18	29	2	0	17	1	0	100	♀	
35	31	8	1912 Jan. 1	2	0	5	3	0	100	♀	



TABLE 1—Continued

NUMBER OF SERIES	DATE OF BIRTH OF YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOLLOW- ING GEN.	NUMBER OF PARENT FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PERCENT OF ♂	PERCENT OF ♀	TYPE	REMARKS
65	1912 Feb. 17	20	1912 Feb. 18	1	0	17	3	0	100	S	
66	20	13	20	1	0	10	3	0	100	S	
67	21	16	21	1	0	16	1	0	100	S	
68	23	9	24	3	0	9	0	0	100	S	
69	26	3	26	2	0	3	0	0	100	S	
70	28	21	29	5	0	19	2	0	100	S	
71	Mar. 1	20	Mar. 2	2	2	18	0	10	90	S	
72	3	20	3	1	0	19	1	0	100	S	
73	3	19	6	5	0	18	1	0	100	S	
74	8	21	8	2	4	16	1	20	80	S	
75	10	8	10	1	0	6	2	0	100	S	
76	12	6	12	1	0	6	0	0	100	S	
77	13	22	13	1	1	20	1	5	95	S	
78	15	15	15	1	0	15	0	0	100	S	
79	16	20	16	1	0	19	1	0	100	S	
80	18	22		1	0	21	1	0	100	S	Disc.
<i>History of Series J</i>											
1	1911 Dec. 2	1									
2	4	9	Dec. 4	2	1	2	6	33	67	S	Prod. H
3	6	6	6	1	0	5	1	0	100	H	
4	8	18	8	1	2	16	0	11	89	H	
5	9	18	10	4	1	17	0	6	94	H	
6	12	10	12	1	0	10	0	0	100	I	







4	Dec.	8	Dec.	8	1	2	12	6	14	86	H
5	9	9	9	9	1	0	15	0	0	100	H
6	10	10	10	10	1	0	16	0	0	83	H, I
7	11	11	11	11	5	0	17	2	17	100	I, H
8	12	12	12	12	2	0	18	0	0	100	H
9	13	13	13	13	2	0	19	0	0	100	H
10	14	14	14	14	1	0	20	0	0	100	H
11	15	15	15	15	1	0	21	0	0	100	H
12	16	16	16	16	1	0	22	0	0	100	H
13	17	17	17	17	1	0	23	0	0	100	H
14	18	18	18	18	1	0	24	0	0	100	H
15	19	19	19	19	1	0	25	0	0	100	H
16	20	20	20	20	1	0	26	0	0	100	H
17	21	21	21	21	2	0	27	0	0	100	H
18	22	22	22	22	2	0	28	0	0	100	H
19	23	23	23	23	1	0	29	0	0	100	H
20	24	24	24	24	1	0	30	0	0	100	H
21	25	25	25	25	1	0	31	0	0	100	H
22	26	26	26	26	1	0	32	0	0	100	H
23	27	27	27	27	1	0	33	0	0	100	H
24	28	28	28	28	1	0	34	0	0	100	H
25	29	29	29	29	1	0	35	0	0	100	H
26	30	30	30	30	1	0	36	0	0	100	H
27	31	31	31	31	1	0	37	0	0	100	H
28	32	32	32	32	1	0	38	0	0	100	H
29	33	33	33	33	1	0	39	0	0	100	H
30	34	34	34	34	1	0	40	0	0	100	H
31	35	35	35	35	1	0	41	0	0	100	H
32	36	36	36	36	1	0	42	0	0	100	H
33	37	37	37	37	1	0	43	0	0	100	H
34	38	38	38	38	1	0	44	0	0	100	H
35	39	39	39	39	1	0	45	0	0	100	H
36	40	40	40	40	1	0	46	0	0	100	H
37	41	41	41	41	1	0	47	0	0	100	H
38	42	42	42	42	1	0	48	0	0	100	H
39	43	43	43	43	1	0	49	0	0	100	H
40	44	44	44	44	1	0	50	0	0	100	H
41	45	45	45	45	1	0	51	0	0	100	H
42	46	46	46	46	1	0	52	0	0	100	H
43	47	47	47	47	1	0	53	0	0	100	H
44	48	48	48	48	1	0	54	0	0	100	H
45	49	49	49	49	1	0	55	0	0	100	H
46	50	50	50	50	1	0	56	0	0	100	H
47	51	51	51	51	1	0	57	0	0	100	H
48	52	52	52	52	1	0	58	0	0	100	H
49	53	53	53	53	1	0	59	0	0	100	H
50	54	54	54	54	1	0	60	0	0	100	H
51	55	55	55	55	1	0	61	0	0	100	H
52	56	56	56	56	1	0	62	0	0	100	H
53	57	57	57	57	1	0	63	0	0	100	H
54	58	58	58	58	1	0	64	0	0	100	H
55	59	59	59	59	1	0	65	0	0	100	H
56	60	60	60	60	1	0	66	0	0	100	H
57	61	61	61	61	1	0	67	0	0	100	H
58	62	62	62	62	1	0	68	0	0	100	H
59	63	63	63	63	1	0	69	0	0	100	H
60	64	64	64	64	1	0	70	0	0	100	H
61	65	65	65	65	1	0	71	0	0	100	H
62	66	66	66	66	1	0	72	0	0	100	H
63	67	67	67	67	1	0	73	0	0	100	H
64	68	68	68	68	1	0	74	0	0	100	H
65	69	69	69	69	1	0	75	0	0	100	H
66	70	70	70	70	1	0	76	0	0	100	H
67	71	71	71	71	1	0	77	0	0	100	H
68	72	72	72	72	1	0	78	0	0	100	H
69	73	73	73	73	1	0	79	0	0	100	H
70	74	74	74	74	1	0	80	0	0	100	H
71	75	75	75	75	1	0	81	0	0	100	H
72	76	76	76	76	1	0	82	0	0	100	H
73	77	77	77	77	1	0	83	0	0	100	H
74	78	78	78	78	1	0	84	0	0	100	H
75	79	79	79	79	1	0	85	0	0	100	H
76	80	80	80	80	1	0	86	0	0	100	H
77	81	81	81	81	1	0	87	0	0	100	H
78	82	82	82	82	1	0	88	0	0	100	H
79	83	83	83	83	1	0	89	0	0	100	H
80	84	84	84	84	1	0	90	0	0	100	H
81	85	85	85	85	1	0	91	0	0	100	H
82	86	86	86	86	1	0	92	0	0	100	H
83	87	87	87	87	1	0	93	0	0	100	H
84	88	88	88	88	1	0	94	0	0	100	H
85	89	89	89	89	1	0	95	0	0	100	H
86	90	90	90	90	1	0	96	0	0	100	H
87	91	91	91	91	1	0	97	0	0	100	H
88	92	92	92	92	1	0	98	0	0	100	H
89	93	93	93	93	1	0	99	0	0	100	H
90	94	94	94	94	1	0	100	0	0	100	H
91	95	95	95	95	1	0	101	0	0	100	H
92	96	96	96	96	1	0	102	0	0	100	H
93	97	97	97	97	1	0	103	0	0	100	H
94	98	98	98	98	1	0	104	0	0	100	H
95	99	99	99	99	1	0	105	0	0	100	H
96	100	100	100	100	1	0	106	0	0	100	H
97	101	101	101	101	1	0	107	0	0	100	H
98	102	102	102	102	1	0	108	0	0	100	H
99	103	103	103	103	1	0	109	0	0	100	H
100	104	104	104	104	1	0	110	0	0	100	H
101	105	105	105	105	1	0	111	0	0	100	H
102	106	106	106	106	1	0	112	0	0	100	H
103	107	107	107	107	1	0	113	0	0	100	H
104	108	108	108	108	1	0	114	0	0	100	H
105	109	109	109	109	1	0	115	0	0	100	H
106	110	110	110	110	1	0	116	0	0	100	H
107	111	111	111	111	1	0	117	0	0	100	H
108	112	112	112	112	1	0	118	0	0	100	H
109	113	113	113	113	1	0	119	0	0	100	H
110	114	114	114	114	1	0	120	0	0	100	H
111	115	115	115	115	1	0	121	0	0	100	H
112	116	116	116	116	1	0	122	0	0	100	H
113	117	117	117	117	1	0	123	0	0	100	H
114	118	118	118	118	1	0	124	0	0	100	H
115	119	119	119	119	1	0	125	0	0	100	H
116	120	120	120	120	1	0	126	0	0	100	H
117	121	121	121	121	1	0	127	0	0	100	H
118	122	122	122	122	1	0	128	0	0	100	H
119	123	123	123	123	1	0	129	0	0	100	H
120	124	124	124	124	1	0	130	0	0	100	H
121	125	125	125	125	1	0	131	0	0	100	H
122	126	126	126	126	1	0	132	0	0	100	H
123	127	127	127	127	1	0	133	0	0	100	H
124	128	128	128	128	1	0	134	0	0	100	H
125	129	129	129	129	1	0	135	0	0	100	H
126	130	130	130	130	1	0	136	0	0	100	H
127	131	131	131	131	1	0	137	0	0	100	H
128	132	132	132	132	1	0	138	0	0	100	H
129	133	133	133	133	1	0	139	0	0	100	H
130	134	134	134	134	1	0	140	0	0	100	H
131	135	135	135	135	1	0	141	0	0	100	H
132	136	136	136	136	1	0	142	0	0	100	H
133	137	137	137	137	1	0	143	0	0	100	H
134	138	138	138	138	1	0	144	0	0	100	H
135	139	139	139	139	1	0	145	0	0	100	H
136	140	140	140	140	1	0	146	0	0	100	H
137	141	141	141	141	1	0	147	0	0	100	H
138	142	142	142	142	1	0	148	0	0	100	H
139	143	143	143	143	1	0	149	0	0	100	H
140	144	144	144	144	1	0	150	0	0	100	H
141	145	145	145	145	1	0	151	0	0	100	H
142	146	146	146	146	1	0	152	0	0	100	H
143	147	147	147	147	1	0	153	0	0	100	H
144	148	148	148	148	1	0	154	0	0	100	H
145	149	149									

TABLE 1—Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF SECOND YOUNG	NUMBER OF INDIVID- UALS OF PARENT IN PAIR	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PERCENT OF ♂	PERCENT OF ♀	TYPE	REMARKS
33	1912 Jan. 27	8	1912 Jan. 27	1	5	3	0	63	37	H	
34	28	16	28	1	6	10	0	37	63	H	
35	30	16	30	1	10	6	0	63	37	H	
36	Feb. 1	21	Feb. 1	1	5	15	1	25	75	H	
37	4	3	4	1	2	1	0	66	33	H	
38	6	15	6	1	0	14	1	0	100	H	
39	8	20	9	3	2	18	0	0	100	H	
40	10	23	10	1	1	19	3	5	95	H	
41	12	18	12	3	2	14	2	12	88	H	
42	14	10	15	4	4	6	0	40	60	H	
43	17	8	17	1	5	3	0	62	37	H	
44	18	15	18	1	0	15	0	0	100	H	
45	20	10	21	3	5	4	1	55	45	H	
46	23	8	23	1	0	6	2	0	100	H	
47	25	16	26	3	4	11	1	26	74	H	
48	28	21	28	1	16	5	0	76	24	H	
49	Mar. 1	17	1	2	7	9	1	44	56	H	
50	3	18	3	2	10	5	3	67	33	H	
51	5	12	5	1	4	8	0	33	67	H	
52	7	10	7	1	0	9	1	0	100	H	
53	9	13	9	2	5	8	0	39	61	H, I	
54	11	8	11	1	2	5	1	29	71	H	
55	13	21	13	1	6	15	0	24	76	S, H	
56	14	18	16	8	9	8	1	53	47	S, I, H	
57	17	20	17	1	10	10	0	50	50	H	

58	Mar. 18	17	Mar. 19	3	6	9	2	40	60	H
59	20	20	20	1	6	13	1	32	68	H
60	22	22	22	3	2	9	1	18	82	H
61	24	15	24	1	3	8	4	27	73	H
62	26	14	26	1	7	7	0	50	50	H
63	28	20	28	1	12	8	0	60	40	H
64	29	21	29	1	11	10	0	52	48	H
65	30	13	30	3	10	3	0	78	22	H
66	1	20	Apr. 1	1	17	3	0	85	15	H
67	3	21	3	1	17	3	1	85	15	H
68	4	9	5	3	1	8	0	11	89	H
69	7	21	7	1	11	10	0	52	48	H
70	8	22	9	6	18	3	1	85	15	H
71	10	25	10	1	11	13	1	46	54	H
72	12	21	12	1	11	8	2	58	42	H
73	13	18	13	1	8	8	2	50	50	H
74	14	21	14	1	10	10	1	50	50	H
75	16	10	16	1	0	10	0	0	100	H
76	17	18	17	1	11	7	0	61	39	H <sub>1</sub>
77	19	5	21	4	0	5	0	0	100	S <sub>1</sub> T <sub>1</sub> H
78	22	9	22	1	2	7	0	22	78	H
79	25	16	25	1	10	5	1	67	33	H
80	26	20	26	3	15	5	0	75	25	H
81	29	20	29	1	13	7	0	65	35	H
82	30	24	30	1	9	14	1	39	61	H
83	1	7	May 2	4	4	3	0	57	43	H
84	3	22	3	1	10	12	0	45	55	H
85	4	21	4	1	5	13	3	28	72	H
86	5	10	5	1	0	8	2	0	100	H
87	6	6	6	1	1	5	0	16	84	H

Rest disc.

Disc.

The histories of *J* and *K*, on the other hand, are very different and were a great surprise to us at the time the experiment was conducted. The transition from the saccate to the humped type which was so tardy and transient in *H* and *I*, appeared in *K* promptly in the second generation and in *J* in the third. Moreover, when once produced the humped type became singularly constant throughout almost the entire series. But once in *J* and three times in *K* did single generations occur in which a part of the individuals only reverted to the saccate type; yet, though no preference was shown in the selection of humped individuals to continue the series at these times of reversion and although no change of food was made, the humped type was immediately resumed in the next generation.

This constancy of the higher type was in striking contrast to our entire previous experience in the rearing of pedigree series of this rotifer. While in mass culture the humped type is readily maintained yet in no other instance of a long continued series of isolation cultures had it been possible to avoid frequent reversions to the smaller saccate type. The only instance where this was done for a score or more generations<sup>2</sup> was where a special food stimulus was applied. That our success with *J* and *K* was not due to food supply employed is proven by the fact, which we have already suggested, that the copious food supply was taken from the same source as was that fed to *H* and *I* and was given to all in excess of consumption. In size *J* and *K* were of course larger than *H* and *I*, being of the humped type; the average number of young produced per individual approximated closely to fourteen in *J* and to fifteen in *K*.

A study of the metabolic rhythm of the four series, which may be made out by noting the number of young present in successive generations, shows the relative independence of the four series in regard to each other, and also in regard to external conditions. Their crescendos and diminuendos are not coincident. Nevertheless, a closer approach to parallelism obtains between *J* and *K*, on the one hand, and between *H* and *I*, on the other, than obtains between any two of the contrasted series.

<sup>2</sup> See article I, table 10, history of Series *D*.

In order to test further the nature of *H* and *I* in regard to their latent potentiality to produce the higher type, derivative mass cultures were started from time to time, it having been proven by all our previous experience that mass cultures favor the progressive mutation to the larger types. Irregular food supplies were purposely resorted to in these cultures as favorable to the transition. Transitions did indeed occur to the humped type, and in a few instances cannibalistic campanulates also made their appearance. This latter transition, however, was far less frequent than in parallel mass cultures derived from *J* and *K*.

The natural conclusion suggested by the conduct of the above series is that we do have the transmission of experimentally induced factors through the sexual gametes and the resulting resting egg. While this result is not shown morphologically in the generation immediately derived from the zygote yet it would seem to plainly exist as an inherent factor, the factor which we have termed the physiological potential.

In order to accumulate further evidence for the fact of this transmission and also to ascertain whether such transmission is, as it were, a uniform process or whether it may perhaps be variable or cumulative, or regressive, we continued the rearing of further series derived from resting eggs produced by the same stocks.

As the labor of rearing these stocks is very considerable and we wished to mass abundant proof upon the points tested we choose to limit the work to inbred series only.

*Series derived by inbreeding individuals of I and J*

It may be first explained that owing to the diverse inherent tendencies of reproduction it was easy to obtain resting eggs from humped series like *J* while it was a matter of much difficulty to obtain them from saccate series like *I*. It was thus not feasible to begin a new parallel series of these two types simultaneously. Adequate controls however were always conducted. To test the inheritance of the high potential of *J* new series were derived as follows: On January 15, during the twenty-first generation of *J*, a mass culture was obtained from the fourteenth daughter of the

mother in the pedigree line. This culture was treated according to our regular method for inducing male production.<sup>3</sup> The effect was quite successful. Resting eggs followed. On January 20 sister eggs from one individual were isolated under conditions favorable for hatching, which began by February 1. Ten of these young were isolated in cultures normal as to food supply and to all other known conditions. These ten individuals became the parents of ten series which, for brevity, we designate collectively as *J 2*.

The effort was made to parallel *J 2* with series derived from the series *H* or *I*, but as already mentioned, difficulty is always expe-

<sup>3</sup>In an article appearing in *Science*, vol. 38, pp. 786-788, A. Franklin Shull, in discussing this method of determining sex or male production, offers certain objections based upon his study of *Hydatina senta*. In regard to the relation of physiological rhythm, nutrition and male production, we should state that since the last two may be directly controlled or modified by experimental conditions, and since the first may also be modified, though not by direct methods, we have a means whereby we may govern this relation at will. That Shull has healthy lines which pass through long periods of parthenogenetic female production, is not surprising. We have had parallel cases; for instance, one of our healthy lines had given rise to a continuous parthenogenetic female production for over sixty generations. However, conditions were clearly not such that male production was possible, but later when conditions were altered to those favorable to male production, this hitherto total female-producing line suddenly threw a large percentage of male producers. That this was due entirely to some unknown internal factor would seem all but improbable. But even accepting this, would it help in explaining results which have been obtained?

In our study of sex determination we have attempted to emphasize that the individual is the "point of action" rather than a number of generations. A propos to this we would call attention to those cytological facts obtained by Erlanger and Lauterborn (*R. Zool. Anz.*, Bd. 20), Lensen (*Zool. Anz.*, Bd. 21), and Jennings (*Bull. Mus. Comp. Zool.*, Harvard Coll., No. 30), upon *Hydatina* and *Asplancha* which tend to prove that during the period of maturation sex is determined by the casting off of the polar bodies. The reduction in the number of chromosomes occurring when the second polar body is cast off, results in male production, while in ova which cast off only one polar body there results female production. The time at which this maturation occurs corresponds to the time at which the factors we advocate are active in the determination of sex. It would be rather difficult to correlate this evidence, it seems to us, with definite internal causes or to chemicals which act only upon the preceding generation, as is offered in explanation of sex determination by Shull.

rienced in obtaining resting eggs from the saccate type. However, a mass culture started on January 29, from the fifty-third generation of *I* and remaining purely saccate, produced by February 6 resting eggs. These began to hatch by February 12. Unfortunately, at this time the labor of caring for the enormous progeny of the ten lines of *J 2*, as well as of the original series, rendered it impossible to establish more than a single additional series. This, started from one of the before-mentioned resting eggs drawn from the saccate series, *I*, we term series *I 2*. It thus forms a parallel or contrast series to the ten lines in *J 2*. Other individuals derived from sister eggs, as well as from other resting eggs produced at the same time in the same culture, were set aside to form mass cultures useful in a general way as checks or controls. The history of *J 2* as well as of the contrasted series *I 2* are given in table 2.

It will be seen from table 2 that seven of the series in *J 2* were bred for ten generations, the other three succumbing to total male production after six to eight generations. *I 2* was bred to thirty generations.

It will be noted that again sexual reproduction fails to wipe out the induced but hereditary tendencies of the contrasted series. Although conditions were in every way parallel the transition from the saccate to the humped type occurred early in each of the ten lines of *J 2*, while in *I 2* no transition whatever occurred. In reality the contrast is even stronger than indicated by this general statement, for not only is there no tendency toward mutation displayed during the entire thirty generations of *I 2*, but in not one of the above mentioned mass cultures derived from the resting eggs of the same source did the humped type appear. The exact generation, on the other hand, in which the humped type appeared in the lines of *J 2* were, respectively, the ninth, fifth second, third, third, third, fourth, sixth, fourth and third. It is noteworthy that even in the one instance where the transition in the direct line was delayed till the ninth generation sister individuals to the one in the direct line made the transition as early as the third generation.



No. 1

NUMBER OF SERIES	DATE OF BIRTH OF YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS IN FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PERCENT OF ♂	PERCENT OF ♀	TYPE	REMARKS
1	1912 Feb.	1	1912 Feb.	1	0	11	0	0	100	S	Sis. Prod. H
2	2	11	3	1	0	6	2	0	100	S	
3	5	8	5	2	0	3	0	0	100	S	
4	4	3	8	1	0	0	0	0	100	S	
5	9	11	10	2	0	11	1	0	100	S	
6	11	11	12	4	1	9	1	10	90	S	
7	14	7	14	2	3	4	0	43	57	S	
8	16	8	16	1	1	7	0	12	87	S	
9	19	8	19	3	3	4	1	43	57	H	
10	21	6		3	0	6	0	0	100	H	

No. 2

1	1912 Feb.	1	1912 Feb.	1	0	1	1	0	100	S	
2	3	2	3	1	0	3	0	0	100	S	
3	5	3	5	1	0	3	0	0	100	S	
4	4	7	8	4	4	3	1	57	43	S	
5	10	15	10	2	6	9	0	40	60	H	
6	12	9	13	2	0	9	0	0	100	H	
7	14	8	14	1	0	8	0	0	100	H	
8	16	8	16	1	1	6	1	14	86	H	
9	18	14	18	2	1	12	1	8	92	H	
10	19	9		2	5	3	1	68	32	H	

No. 3

1912	1912	1912	Prod. H
Feb.	Feb.	Feb.	
1	1	1	S
2	13	5	H
3	3	11	H
4	8	13	H
5	13	16	H
6	15	18	H
7	18	20	H
8	20	22	H
9	22	24	H
10	24	24	I

Fig. 4

[illegible]

No. 5

		1912																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
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TABLE 2—Continued  
No. 5—Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG OF FIRST YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS IN PARENT IN FAMILY	NUMBER OF ♀	NUMBER OF ♂	NUMBER DEATHS	PER CENT OF ♀	PER CENT OF ♂	TYPE	REMARKS
No. 5											
4	1912 Feb. 8	11	1912 Feb. 10	6	6	5	0	54	46	H	
5	12	10	12	2	0	10	0	0	100	H	
6	14	12	14	1	1	11	0	9	91	H	
7	15	16	16	2	10	6	0	63	37	H	
8	18	12	21	8	7	4	1	64	36	H	
9	23	10	23	1	1	9	0	10	90	H	
10	25	8			0	7	1	0	100	H	
No. 6											
1	1912 Feb. 2	1	1912								
2	4	9	Feb. 5	2	1	8	0	12	87	S	Sis Prod. H
3	8	5	8	1	1	3	1	25	75	H	
4	19	12	19	2	0	11	1	0	100	H	
5	13	6	13	1	2	4	0	33	67	H	
6	15	7	16	3	7	2	0	72	28	H	
7	18	8			7	1	0	87	13	H	
No. 7											
1	1912	1	1912								
2	Feb. 2	5	Feb. 6	1	0	3	2	0	100	S	
3	8	6	9	4	0	4	2	0	100	S	
4	11	3	11	1	0	2	1	0	100	H	

				No. 8							
				1912							
				Feb.							
5	13	13	13	2	1	10	2	9	91	H	
6	15	6.	15	1	0	6	0	0	100	H	
7	18	2	18	1	0	2	0	0	100	H	
8	20	7			7	0	0	100	0	H	
				1912							
				Feb.							
1	5	1	9	3	1	2	1	0	100	S	
2	6	3	10	6	0	12	0	0	0	S	
3	10	12	13	1	0	15	0	0	0	S	
4	13	15	16	3	0	6	0	0	0	S	
5	15	6	16	2	9	8	0	33	47	H	
6	18	17	19	1	8	8	1	50	50	H	
7	20	17	20	1	0	5	1	0	100	H	
8	23	6	23	1	0	9	0	0	0	H	
9	26	9	27	4	0	9	0	94	6	H	
10	28	20			18	1	1				
				1912							
				Feb.							
1	5	1	7	2	2	9	0	18	82	S	
2	6	11	9	2	5	5	0	50	50	S	
3	9	10	10	1	2	8	1	20	80	H	
4	10	11	12	1	10	4	0	70	30	H	
5	12	11	13	1	0	15	0	0	100	H	
6	14	15	14	1	0	6	0	50	50	H	
7	16	12	16	2	6	6	0	0	100	I	
8	18	1	18	1	0	1	0	0	100	I	
9	20	6	20	2	0	6	0	0	100	I	
10	23	10			4	6	0	40	60	H, I	

TABLE 2—Continued  
No. 10

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOUNDED GEN.	NUMBER OF INDIV- IDUALS OF PARENT IN FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PER CENT OF ♂	PER CENT OF ♀	TYPE	REMARKS
1912											
1	Feb. 6	1									
2	9	6	Feb. 9	1	0	6	0	0	100	S	
3	12	6	12	1	2	4	0	33	66	H	
4	14	13	14	1	2	11	0	15	85	H	
5	16	10	16	3	1	8	1	11	89	H	
6	18	5			3	0	2	100	0	H	
History of Series 12, derived from mass culture from the fifty-third generation of I. Parents of saccate type											
1912											
1	Feb. 12	1									
2	17	5	Feb. 17	1	0	5	0	0	100	S	
3	18	17	20	3	0	15	2	0	100	S	
4	21	6	21	1	0	6	0	0	100	S	
5	23	4	23	1	0	3	1	0	100	S	
6	25	5	26	2	0	5	0	0	100	S	
7	28	19	28	1	0	19	0	0	100	S	
8	1	21	Mar. 1	1	4	17	0	19	81	S	
9	2	9	2	1	0	8	1	0	100	S	
10	4	11	5	6	0	11	0	0	100	S	
11	8	5	8	1	0	5	0	0	100	S	
12	10	8	10	2	0	7	1	0	100	S	
13	12	8	12	1	0	6	2	0	100	S	
14	13	13	13	2	0	12	1	0	100	S	

	Mar. 15	Mar. 15	Mar. 15	1	0	13	0	0	0	0	100	5
15	13											S
16	20	16										S
17	18	7		1		19	0		1			S
18	19			1		7	0		0			S
19	22			1		22	0		0			S
20	21	19		1		18	1		0			S
21	23	20		1		20	0		0			S
22	24	19		8		19	0		0			S
23	28	13		3		13	0		0			S
24	29	21		1		21	0		0			S
25	30	6		1		6	0		0			S
26	1	7	Apr.	1		5	0		28	72		S
27	2	2		2		1	0		0	50		S
28	4	12		1		9	0		25	75		S
29	5	16		1		12	0		25	75		S
30	6	21		2		15	0		29	71		S
31	9	21				8	0		62	38		I

It can not be other than surprising to find this all but uniform maintenance of high and low potential respectively in these series of rotifers, carried not only through long series of parthenogenetic reproduction, but through sexual reproduction as well, and this in spite of the fact that sexual reproduction involves the temporary return to the apparently uniform saccate type.

To test the matter still further, through more cycles of sexual reproduction, and also to make up for the paucity of numbers in the series on the saccate side in our last experiment, further experiments were begun as follows: Mass cultures were founded from the tenth and fourteenth generations of the saccate series *I 2*. These were carefully fed so as to induce male production without the customary accompaniment of the transition to the humped type. This end was successfully attained in both cultures. A young female from the mass culture derived from the tenth generation was fertilized, and by March 20 had produced resting eggs. Two of these hatched on March 27, one of which became the parent of a pedigree series termed *I of I 3*. From the mass culture derived from the fourteenth generation of *I 2* more resting eggs were secured. A number were dropped by four individuals on March 12 and 22. These were isolated and from a large number which hatched on March 28 and 29 fourteen were chosen to become the parents of pedigree series, which make up the second part of the group of series termed *I 3*. This group of series thus compose the third group of inbred saccate lines derived by sexual reproduction, each group from the preceding group, and all from the original parthenogenetic individual.

That these series of *I 3* might be paralleled by at least a few series derived from our humped lines two new series were started from *J*. It was perhaps unfortunate that these series could not have been started from some one of the ten series of *J 2*, but these series had been discarded after the end of the tenth generation, as we had not at that time considered the possibility of the prolonged continuance of our experiments. It thus became necessary to have recourse to the original series *J*. On March 15 the tenth individual of the fiftieth generation of *J* was fertilized by a male produced by a sister individual of the same generation. Resting eggs were formed which hatched on March 26. One only was

saved and from it was reared a series which we term *J 2'*. Another series of similar derivation was reared from a resting egg produced by the third individual of the fifty-fourth generation, fertilized by a male, a brother to the one used to fertilize the mother of the preceding series. The series was begun by isolating the individual on March 24. We term this series *J 2''*. The history of the fifteen series of *I 3*, together with the contrasting series *J 2'* and *J 2''*, is given in table 3.

The study of table 3 shows again the surprising fact—the complete hereditary continuence through the resting egg of the diverse qualities originally induced in the two contrasting stocks. Throughout the entire ten generations through which the fifteen series of *I 3* were carried, not a single instance of the transition from the saccate type to the humped type occurred, although in a few of numerous mass cultures derived from these saccate series such transitions did occur, though sparingly. On the other hand, despite uniform feeding and other parallel conditions, the lines *J 2'* and *J 2''* again leapt almost immediately to the humped form. This occurred in the second and third generation respectively, and it is of no less significance that there was in each of these series an almost immediate production, no doubt through cannibalism, of the third or campanulate type. This shows still more conclusively the inherent high potential so fully maintained by this stock.

Although no farther evidence was needed to test the single matter of inherited potential through the resting egg, yet the thought of the possibility of the formation of a saccate race devoid of any mutational tendency induced us to develop still more series. A culture derived from the fourth generation of series *11* of *I 3* was allowed to mass. Males were produced by saccate individuals on April 14 and resting eggs were duly formed by saccate females. An individual, hatched from one of these, gave the starting point of series *I 4*. It was bred through thirteen generations. Its history is given in table 4. Again the saccate type was constant throughout the entire series. But in mass cultures, which were now forced by special feeding to test at once their character, it was still possible to induce the transformation to the humped type. No campanulates were formed.



TABLE 3  
History of Series I 3, derived from mass culture from the tenth generation of I 2. Parents of saccate type. No. 1

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF INDIVIDUALS OF PARENT IN FAMILY	NUMBER OF ♂♂	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♂	PER CENT OF ♀♀	TYPE	REMARKS
1	1912 Mar. 27	1	1912 Mar. 27	1	0	9	1	0	100	S	
2	Mar. 29	10	Mar. 31	2	3	8	2	28	72	S	
3	Mar. 31	13	Apr. 2	1	0	13	1	0	100	S	
4	Apr. 2	14	Apr. 3	1	0	12	1	0	100	S	
5	Apr. 3	13	Apr. 4	1	0	17	1	0	100	S	
6	Apr. 4	18	Apr. 5	1	8	8	0	50	50	S	
7	Apr. 5	16	Apr. 6	1	1	10	1	9	91	S	Disc.
8	Apr. 6	12	Apr. 8	2	0	4	0	0	100	S	Disc.
9	Apr. 8	4			0	20	2	0	100	S	
10	Apr. 10	22			0						

Derived from mass culture from the fourteenth generation of I 2. Parents of saccate type. No. 2

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF INDIVIDUALS OF PARENT IN FAMILY	NUMBER OF ♂♂	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♂	PER CENT OF ♀♀	TYPE	REMARKS
1	1912 Mar. 29	1	1912 Mar. 30	1	1	18	0	5	95	S	
2	Mar. 30	19	Mar. 31	1	0	18	0	0	100	S	
3	Mar. 31	18	Apr. 2	1	0	10	0	0	100	S	
4	Apr. 2	10	Apr. 4	1	0	16	1	0	100	S	
5	Apr. 4	17	Apr. 5	1	2	16	0	11	89	S	
6	Apr. 5	18	Apr. 6	1	2	5	0	28	72	S	
7	Apr. 6	7	Apr. 7	1	0	13	0	0	100	S	
8	Apr. 7	13	Apr. 8	1	1	27	0	36	64	S	
9	Apr. 8	28			0	6	1	0	100	S	
10	Apr. 10	7			0						

No. 3									
1912									
1	Mar. 29	1							
2	Mar. 30	14							
3	Apr. 1	16	1	0	14	0	0	100	S
4	3	18	1	7	10	3	0	100	S
5	4	15	1	3	12	0	41	59	S
6	5	22	3	5	16	0	20	80	S
7	6	21	1	6	13	2	24	76	S
8	7	20	3	4	16	0	32	68	S
9	9	23	1	11	12	0	20	80	S
10	10	21		3	18	0	48	52	S
							15	85	S
No. 4									
1912									
1	Mar. 30	1		3	13	0	19	81	S
2	31	8	1	0	7	1	0	100	S
3			1	0	18	2	10	90	S
4	Apr. 2	22	1	2	9	0	18	82	S
5	3	11	1	2	13	1	46	54	S
6	4	25	1	11	15	0	25	75	S
7	5	20	1	5	17	0	6	94	S
8	6	18	1	1	16	1	6	94	S
9	8	18		1	16	1	30	70	S
10	10	23		7	16	0			S
No. 5									
1912									
1	Mar. 29	1		0	4	2	0	100	S
2	31	6	1	0	13	1	0	100	S
3	Apr. 1	14							

TABLE 3—Continued  
No. 5—Continued

NUMBER OF SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS IN PARENTIN FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER DEATHS	PER CENT OF ♂	PER CENT OF ♀	TYPE	REMARKS
4	1912 Apr. 4	11	1912 Apr. 4	1	2	9	0	18	82	S	
5	5	7	5	1	0	7	0	0	100	S	
6	6	5	6	1	1	4	0	20	80	S	
7	8	3	8	1	2	1	0	67	33	S, I	
8	9	8	9	1	4	4	0	50	50	I, S	
9	10	21	10	1	0	21	0	0	100	S	
10	11	20	11	1	0	20	0	0	100	S	
No. 6											
1	1912 Mar. 29	1	1912 Mar. 30	1	0	4	0	0	100	S	
2	30	4	1	1	0	18	0	0	100	S	
3	1	18	1	1	0	5	2	0	100	S	
4	2	7	2	1	0	15	2	0	100	S	
5	4	17	4	2	0	13	0	0	100	S	
6	5	13	5	1	0	4	0	20	80	S	Rest disc.
7	6	5	7	4	1	4	0	0	100	S	Rest disc.
8	8	4	8	1	0	4	0	0	100	S	
9	4	19	9	1	5	13	1	28	72	S	
10	11	17		1	0	15	2	0	100	S	

No. 7									
1912									
1	2	3	4	5	6	7	8	9	10
Mar. 29	Mar. 30	Mar. 31	Apr. 4	Apr. 5	Apr. 6	Apr. 8	Apr. 10	Apr. 11	Apr. 12
1	16	17	15	21	24	21	13	18	20
1	8	1	1	1	1	1	1	1	1
1	1	0	1	5	0	8	6	1	2
15	17	13	16	23	13	7	16	18	
0	0	0	0	1	0	0	1	0	
6	0	7	24	0	38	46	6	10	
94	100	93	76	100	62	54	94	90	
S	S	S	S	S	S	S	S	S	
No. 8									
1912									
1	2	3	4	5	6	7	8	9	10
Mar. 29	Mar. 30	Mar. 31	Apr. 2	Apr. 3	Apr. 5	Apr. 6	Apr. 8	Apr. 10	Apr. 11
1	26	14	11	23	13	9	4	22	17
1	1	1	1	1	1	1	1	1	1
1	0	0	1	5	4	0	1	0	0
26	12	10	18	9	8	3	21	15	
0	2	0	0	0	1	0	0	2	
0	0	9	22	30	0	25	5	0	
100	100	91	78	70	100	75	95	100	
S	S	S	S	S	S	S	S	S	
No. 9									
1912									
1	2	3	4	5	6	7	8	9	10
Mar. 29	Mar. 30	Mar. 31	Apr. 1	Apr. 2	Apr. 3	Apr. 4	Apr. 5	Apr. 6	Apr. 7
1	18	12	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
17	17	5	0	0	0	0	0	0	0
6	37	4							
94	62								
S	S								







No. 16

1912

1912

1	Mar. 28	1	Mar. 30	1	2	17	1	11	89	S
2	Mar. 30	20	Mar. 31	1	0	19	0	0	100	S
3	Mar. 31	19	Apr. 1	1	5	21	0	24	76	S
4	Apr. 1	26	Apr. 2	1	2	17	0	12	88	S
5	Apr. 2	19	Apr. 4	1	1	9	0	10	90	S
6	Apr. 4	10	Apr. 5	1	14	6	0	70	30	S
7	Apr. 5	20	Apr. 6	1	3	12	0	20	80	S
8	Apr. 6	15	Apr. 8	1	9	10	0	47	53	S
9	Apr. 8	19		1	4	13	2	23	77	S
10	Apr. 9	19								

History of Series J 2', derived from tenth individual of the fifth generation of J. Parents of humped type.

1912

1912

1	Mar. 25	1	Mar. 28	4	3	4	0	43	57	I, C, H
2	Mar. 27	7	Mar. 30	1	0	6	0	0	100	C
3	Mar. 30	6	Apr. 1	1	0	3	+	0	100	C, H
4	Apr. 1	3	Apr. 3	1	0	4	0	0	100	H, C
5	Apr. 3	4	Apr. 5	1	3	5	0	37	63	H
6	Apr. 5	8	Apr. 6	1	4	11	1	26	74	H
7	Apr. 6	16	Apr. 8	1	2	4	0	33	67	H, C
8	Apr. 8	6	Apr. 10	1	21	5	0	81	19	H
9	Apr. 10	26			17	4	1	81	19	H
10	Apr. 11	22								

History of Series J 2''

1912

1912

1	Mar. 24	1	Mar. 25	1	9	7	1	0	100	S
2	Mar. 25	8	Mar. 29	3	3	4	1	43	57	H, C
3	Mar. 28	S								

Young eaten



TABLE 3—Continued  
*History of Series J 2\*—Continued*

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FROM WHICH ING GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PER CENT OF ♂	PER CENT OF ♀	TYPE	REMARKS
4	1912 Mar. 30	18	1912 Mar. 30	1	1	17	0	6	94	S, I	
5	81 Apr. 2	14	31	1	3	11	0	21	79	H	
6	Apr. 2	25	Apr. 2	1	10	14	1	42	58	H	
7	4	18	4	1	10	6	2	63	37	H	
8	5	25	5	1	7	18	0	28	72	H	
9	7	22	7	1	14	8	0	64	36	H	
10	9	27			10	17	0	37	63	H	

TABLE 4  
History of Series I<sub>4</sub>, derived from a mass culture from the fourth generation of Series 13 of I 8. Parents of saccate type

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF PARENTS OF FAMILY	NUMBER OF ♂♂	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♂	PER CENT OF ♀♀	TYPE	REMARKS
	1912		1912								
1	Apr. 16	1									
2	Apr. 16	3	Apr. 16	1	0	3	0	0	100	s	
3	21	13	21	1	0	13	0	0	100	s	
4	23	15	23	2	0	15	0	0	100	s	
5	25	12	25	1	0	12	0	0	100	s	Rest disc.
6	26	8	26	3	0	8	0	0	100	s	
7	28	20	28	1	0	20	0	0	100	s	
8	30	18	30	1	1	16	1	6	94	s	
9	May 1	7	May 1	1	3	4	0	43	57	s	Rest disc.
10	May 2	14	May 2	1	0	14	0	0	100	s	
11	3	19	3	1	0	19	0	0	100	s	
12	4	2	4	1	0	2	0	0	100	s	Rest disc.
13	5	20			0	19	1	0	100	s	

It was difficult to ascertain whether the tendency to remain in the saccate form was a cumulative one or whether it was remaining constant throughout the experiment. Perhaps the most obvious sign of the decreasing potential was the fact that it was becoming more difficult to produce male production in the saccate series.

In connection with male production, in *I 4*, there occurred, too, for the first time, an interesting phenomenon which points with some probability toward the possibility of a transition to a lower type than the ordinary saccate *A. amphora*. This was the production of a rather unusual number of males, among which a number appeared like transitional forms between the males of *A. amphora* and *A. brightwelli*. By isolating mothers about to produce males it was discovered that these unusual individuals were the early members born in male families; their humps were small or almost lacking, the body was much smaller and more compact than normal, and the corona was much narrowed. All these characters suggest the males of the smaller species. Transitional males were found in all stages between this much reduced type and the customary type of *A. amphora*.

One more series of saccates was bred, which we name *I 5*. It was started in the usual way by resting eggs secured from mass culture, which latter in its turn, had been derived from the seventh generation of *I 4*. This last series of saccate rotifers was bred for ten generations (table 5). It showed every evidence of possessing the full vigor of its predecessors and was likewise perfectly true to type so long as bred under the customary and constant conditions. A mass culture founded from the fifth generation failed entirely to throw the humped type. It again produced a small number of the transition-like males. Last of all, in the seventh and eighth generations, the series was itself again tested, by forced and alternate feeding, to see whether the potentiality of the humped type was still present. Mutation was indeed induced showing that the long hereditary transmission, sexual as well as asexual, of the simple saccate form had not produced a race sufficiently stable to resist the cause for change which lies in an unwonted food stimulus.

It is natural to ask whether any tendency toward such race formation had manifested itself throughout the entire succession



these lines were very large and showed much vigor. This last experiment with humped lines not only adds one more instance of unbroken heredity through the resting egg, but is of interest in that the stock was derived from series *K* rather than from *J*, showing that the two series comport themselves quite alike in this respect. The origin and conduct of the mass culture from which *K* 2 was derived is perhaps of still more interest. It shows that a single isolated parthenogenetic reversion to the saccate type does not necessarily mean a permanent drop in potential to the saccate level. Why, we can not say, but may easily conjecture that the isolated appearance of such saccates may have been due to a somatic rather than a germinal influence.

#### CONCLUSION

We need hardly repeat, in résumé, the main conclusion to which these experiments lead: The hereditary transmission of induced germinal modification through sexual as well as through parthenogenetic generations. Certain minor aspects of the matter, however, need further accentuation. First, the unique fact that a certain type or form when once induced (the humped form of *A. amphora*) may in reality be transmitted with full force despite an interruption of one or more generations (saccates from resting eggs) in which this form is not expressed. Although the conditions are totally different the phenomenon suggests that of recessive and dominant characters.

Second, it is worth while to accentuate the nature of the results of sexual reproduction in this rotifer with regard to vitality and variation. The limits of our experiments, it is true, are narrow, as all of our series were begun with inbreeding. But within these limits the results are definite and uniform. The union of gametes has little if any influence upon the vitality of the race; it is true that the resting eggs hatch irregularly but once hatched they show the normal growth and reproductive energy of the strain from which they are derived. There is no added vitality, no rejuvenation. As to variation again, the results of fertilization seem purely negative, excepting of course, the temporary return to

the saccate form already mentioned. In many respects the conduct of this rotifer when bred in pedigree series suggests parallelism with Protozoa under like handling. But the variation, so often degenerative, which Jennings has shown to result from conjugation in *Paramecium*, finds no parallel within our experiments, so far as we have carried them. On the contrary, sexual reproduction and parthenogenesis seem very closely related and all but equivalent processes.

We may perhaps note in this connection that we have observed a number of instances of simultaneous development of resting eggs and the active young males within the body of the same parent; and what is still more striking in these cases of mixed reproduction, certain of the earlier developing embryos were plainly covered with thin though characteristic coats resembling the resting egg. In some instances such embryos were proven to become females and thus undoubtedly represent fertilized eggs which because of special conditions—possibly the thinner egg coats—were proceeding to immediate development within the uterus quite as do the ordinary parthenogenetic ova. In any case the close relationship of the different types of reproduction was strongly called to mind by the observation of females bearing simultaneously the fully-formed resting eggs, active young males approaching the birth period, and the above mentioned intermediate type of reproductive body.

As to the bearing of these conclusions, it is plain that they throw much light upon our own previous experiments with this rotifer, and, we think, some as well upon the extended experiments of others upon the rotifer, *Hydatina senta*. Thus in our own previous work, entire lines of rotifers started from resting eggs chosen at random, often proved perplexingly dissimilar. They possessed what we now call different potentials, some tending strongly toward the maintenance of the saccate type, some toward the maintenance of the humped type, while others maintained this form plus the occasional production of the campanulate type. It is plain now, after the experiments recorded in this paper, that these contrasting lines of *A. amphora* may have differed solely because of a different parentage of the resting eggs

from which they were started. The frequent outburst, within certain lines, of the mutational tendency in the early generations, which we explained in our former paper as probably due to qualitative nutritional change, we now see may well be due to inherited high potential, with or without the special food stimulus.

Indirectly too, the question of the percentage of male production in different lines meets with a much needed additional explanation. Male production, as we showed in our previous paper, requires as its first and most fundamental condition, a state of high potential. It thus follows naturally that strains originating from resting eggs produced by parents in which this high potential had been built up by special nutrition, or had been inherited parthenogenetically, should become at once strains producing many males; whereas, conversely, strains from resting eggs dropped by parents of low potential inherit and maintain this same inner condition, and while perfectly healthy, produce under normal conditions few males.

In regard to the work of others upon the rotifer *Hydatina*, we find that Punnett, Whitney, and Shull, all in one way or another emphasize the fact of races or strains in their material. These strains of *Hydatina* show no morphological differences but contrast with each other in regard to number of males produced or in regard to the general rate of parthenogenetic reproduction. Both Shull and Whitney have also shown that the qualities of their strains may be transmitted through the resting egg. We believe, however, that in no case have they traced their strains to a definite origin or shown that any quality induced by a known agent became hereditary. Some of the work upon *Hydatina* produces the general impression of a species, split up into what one might be tempted to call 'elementary' strains, akin, in their fixity if not in their morphological differences, to Jennings' races of *Paramecia*; other portions of their work suggest, on the other hand, that these strains are less fixed and in some cases arise while the animals are under experimental observation. We hope that our work may throw some light upon the method of their origin, on their varying degrees of stability, and upon their frequent assumption of a hereditary character.

Lastly, we find in our results suggestions toward the explanation of many of the puzzling diversities of condition in which *Asplanchna amphora* and allied rotifers have been found in nature. We will not enter into details. But it is worth pointing out that some of the systematic students of the genus seem to have known only the saccate type; a number have now found, in at least feeble development, the rotifer in its dimorphic condition, that is, the saccate type giving rise to the humped form; while it seems doubtful at the present time whether the trimorphic condition, which is so copiously present in our vicinity, has as yet been met with elsewhere. After making full allowance for the different methods of study, and so forth, it seems highly probable that this rotifer and its immediate allies exist in nature in different degrees of development in different localities. Under the least favorable conditions it is possible that the saccate type only has been produced. Under more favorable conditions the potential has been raised and inheritable germinal variations have been induced, making possible the regular production of the humped type. Much more rarely, under conditions favoring the development of the species in unusual numbers, the striking though less fixed type which we call the campanulate has resulted. This type transmits its form, never to all, but always to a part of its offspring.

In the brief period of our experiments—about eight months—we were unable to produce types which were stable save when bred under approximately uniform conditions. Extreme starvation or sudden change in opulent nutrition would bring about a reversion to a simpler or stimulate to a higher type. In so far, our work falls short of offering conclusive evidence that the processes which we have described are related to species formation. Nevertheless it is easy to imagine that similar steps may now and then become more permanent in nature. In any case, it is interesting to know that the marked morphological changes induced by nutrition are not confined within the bounds of parthenogenetic series only, but, as far as our experiments show, are equally transmitted by the sexual process. This it seems to us, renders it at least not improbable that the mutational changes shown by this rotifer are phases of a true species-making process.



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# CERTAIN RELATIONS BETWEEN RHEOTAXIS AND RESISTANCE TO POTASSIUM CYANIDE IN ISOPODA<sup>1</sup>

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Earlier experiments (Allee '12-'13) showed that certain conditions known to affect the rate of metabolism of animals regularly affected the rheotactic reaction of the isopod, *Asellus communis*, Say. Low oxygen, chlorotone, potassium cyanide, lowered temperature, suddenly heightened temperature, increased carbon dioxide tension, and starvation, all of which depress the rate of metabolism of animals, also lower the positive rheotactic response of isopods. High oxygen, caffeine, and a gradual increase in temperature have the opposite effect.

A desire to obtain more exact information regarding this correlation of metabolic rate and the rheotactic reaction of isopods led to an attempt to measure the rate of metabolism by means of the survival-time in potassium cyanide. This method was worked out by Professor Child ('13) for planarians, and is essentially as follows:

In a relatively strong solution of potassium cyanide (0.001 mol. or thereabouts) the animals having the highest rate of metabolism die first, or in other words, they have the shortest survival-time. Those having the lowest rate die last and hence have the highest rate of metabolism. Child called this the 'direct method' of

<sup>1</sup> The experiments upon which this paper is based were performed in the Laboratory of Plant Physiology at the University of Illinois and in the Marine Biological Laboratory, Woods Hole, Mass. I am indebted to Dr. C. F. Hottes and Prof. F. R. Lillie for furnishing facilities for carrying on the experiments. I am also indebted to Prof. C. M. Child and to Dr. Shiro Tashiro for many helpful suggestions, and to Marjorie Hill Allee for assistance with certain experiments and for aid in tabulating results.

measuring the resistance of the animal. The effect is due to the action of the cyanide which (Geppert '99) decreases the ability of the tissues to take up oxygen. Child ('13 a) suggests that the effect of the potassium cyanide solution depends upon the number of chemical bonds in the organism which are opened in a given length of time. This means that the higher the rate of reaction, the greater is the opportunity of the cyanide to produce its effect. In a much weaker solution (0.00001 mol.) acclimatization occurs and the animals with the higher rate of reaction live longer than those with a lower rate. This is called the 'indirect method.'

Although Professor Child found this method held for planarians it did not necessarily follow that the chitinous-covered, gill-breathing isopods would be affected in the same manner. My problem then was, first, to find whether or not the cyanide method would hold for isopods and then whether or not there was a relation between the rheotactic reaction and the resistance to potassium cyanide.

#### THE STOCK

Three groups of *Asellus communis* were tried in these experiments:

I. The offspring of a stock that was originally collected in August, 1911, from County Line Creek, north of Glencoe, Illinois (Shelford '11 a, maps) and which had been in well-aerated laboratory aquaria since (Allee '13). These isopods were half-grown when used.

II. A large number of breeding isopods collected in the above stream April 13, 1913, and kept under the same conditions as the first group. Both adults and young were tested.

III. A series of collections of pond isopods, also *A. communis* but mainly juvenile, from small fresh-water ponds near Woods Hole, Massachusetts. These came from silt- and débris-bottomed ponds, low in oxygen. They were kept under similar conditions in the laboratory.

These groups will be referred to in the body of the paper as Stocks I, II and III respectively. The mortality rate of all three groups was low while under observation. During the course of

these experiments individual tests were made of the reactions of 257 isopods of the first two groups, and of 195 of the third lot, making a total of 452 isopods. Moreover, these experiments consistently support results of the preceding four years experimental study on this subject.

#### METHODS

The isopods were tested for their rheotactic reaction in a circular pan the bottom of which was covered with wax. The responses of an individual isopod for ten successive one-minute reaction periods were taken as a fair indication of the rheotactic tendencies of the animal. The approximate distance covered during each reaction was recorded and will be found in a standardized form in the tables under the head of efficiency in the current.<sup>2</sup>

Immediately following the rheotaxis test the animals were placed in potassium cyanide solution in Erlenmeyer flasks of 150 to 2000 cc. capacity. The size of the flask depended upon the amount of oxygen to which the isopod was accustomed and upon the strength of cyanide used. In all instances, had the cyanide been omitted the isopods would have shown no ill effects of confinement for several days. A 0.1 molecular solution of potassium cyanide was made up in distilled water. From this, solutions of the desired strength were made in water similar to that in which the isopods had been kept. During the survival periods in the flasks the temperature was the same as in the stock aquarium.

Exact determination of the death point gave considerable difficulty. It was found that close examination with a lens for

<sup>2</sup> The following arbitrary standard of efficiency in the current was adopted in the earlier experiments and is used in those reported here. This represents numerically the distance covered by an isopod in a minute's reaction period:

- 0 no reaction
- 1 slight movement
- 2 any response between 1 and 3
- 3 progress one-third around the pan positive (approximately 27 cm.) or two-thirds, negative (51 cm.)
- 4 progress two-thirds around the pan positive or one and one-third negative
- 5 progress once around the pan positive or twice around, negative
- 6 any distance over 5

For further details see Allee '13.

movement of the gills or for slight twitching of the anterior legs gave the most satisfactory results. About the time of death the pleopods which act as gill covers move out until they stand almost at right angles with the body and the gills become more or less swollen. As a check upon the supposed death-time, observations were carried on for an interval after the animals were apparently dead. In doubtful cases or with very small isopods, the animals were removed from the flask with a large pipette and examined in a watch-glass under a dissecting microscope. This was never done until movement had almost ceased and the stimulation did not appreciably affect results.

#### TESTING THE CYANIDE RESISTANCE METHOD

If the resistance to potassium cyanide is a measure of the metabolic condition of isopods, then, other things being equal, the higher the temperature the greater the activity in the cyanide, and the younger the animal the sooner it should die. Also the greater the output of carbon dioxide, the shorter should be the survival time. That all these requirements are fulfilled by the cyanide method with isopods is shown by the tests recorded in table 1.

Raising the temperature of isopods during the survival period gave a very marked decrease in the survival time. In making the tests recorded in table 1, the isopods were placed in 0.001 molecular potassium cyanide solution at the same temperature as the water in which they were tested for their rheotactic reaction (18–21°C.). As a control, other isopods as nearly similar as could be selected were placed in an equal amount of water. Both lots were then gradually heated to 30 to 32°. In all cases the isopods in cyanide died in from thirty-five minutes to an hour and forty minutes, a length of time much below the average survival-time of similar isopods in cyanide of the usual temperature. Furthermore, in all instances the isopods in water survived the heating process without being visibly affected by it.

In other experiments an attempt was made to keep isopods at about 25°C. (5 to 8° above the temperature of the stock at that

time) for a week before killing them in cyanide at the same temperature. Under these conditions there was a shortened survival-time but the variations in temperature were too great for the results to be anything but indicative.

In the work with lowered temperature the isopods had been in a temperature of 17 to 19° and were placed in an ice-chest with the temperature at 8 to 14°. After a week under these conditions they were killed in potassium cyanide, 0.0002 molecular solution, at the same low temperature. The isopods at the usual temperature (table 1) showed a higher rate of positive rheotaxis and of efficiency in the current than did those from the lower temperatures and hence would be expected to give a shorter survival-time regardless of the temperature change, but the difference is entirely too great to be altogether accounted for in this way.

It is to be expected that the chemical activity of the cyanide at low temperature would be less than at a higher temperature, but it is highly improbable that the great differences in survival-time at the different temperatures could be due to this cause

TABLE 1

*Showing the effect of different factors upon the survival in potassium cyanide with a view to testing whether the resistance to cyanide bears any relation to the metabolic condition of isopods*

STOCK	EXPERIMENTAL CONDITIONS	MOLECULAR STRENGTH OF KCN SOLUTION	NUMBER ISOPODS USED	AVERAGE RHEOTAXIS FACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS			AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	AVERAGE SURVIVAL- TIME IN HOURS AND MINUTES	RANGE OF TEMPERA- TURE
				+	-	0				
III	Heightened temperature	0.001	10	42	51	7	2.2	5.0	1.03	30-32
III	Usual temperature.....	0.001	55	50	37	10	3	2.2	6.0	5.14 18-21
II	Lowered temperature....	0.0002	9	46	35	19	2.2	12.4	30.17	8-14
I-II	Usual temperature.....	0.0002	72	61	28	11	2.5	10.7	5.59	17-21
II	Stimulation by shaking.	0.0002	6					15.5	2.19	17-21
II	Not stimulated: control	0.0002	9					14.8	5.12	17-21
I-II	Not stimulated: average	0.0002	72	61	28	11	2.5	10.7	5.59	17-21
III	Stimulated by shaking..	0.001	9	31	32	32	5	1.6	4.4	2.39 18-19
III	Not stimulated: average	0.001	55	50	37	10	3	2.2	6.0	5.14 18-21
I-II	12 or more mm. long....	0.0002	20	59	27	14	2.3	14.2	6.25	17-21
I-II	8 or less mm. long.....	0.0002	20	60	23	17	2.4	7.4	5.04	17-21

alone. Moreover, the effect of temperature upon metabolism will adequately explain the difference found to exist. Therefore the results of measuring the survival-time of isopods at low and at high temperatures, while not a complete demonstration, strongly indicate that the resistance to potassium cyanide is a measure of metabolic rate in isopods.

In the case of stimulation of the isopods by shaking them while exposed to the cyanide, there is a marked decrease in the survival-time. This is what would be expected if the resistance to potassium cyanide is a measure of metabolic activity. In these experiments the isopods were shaken every few minutes as long as this stimulated them to activity. If objection be made that shortening of the survival-time under these conditions is a fatigue response, it is only necessary to point out that fatigued animals would have a lower rate of metabolism and hence a longer survival time than unstimulated animals. Consequently, if fatigue enters as a factor, it tends to lengthen, not shorten, the survival-time. But it is improbable that fatigue is a factor in the survival-time of these stimulated isopods since they were usually lying on their backs unable to respond to stimulation before either the pond or stream mores would have shown a change in their rheotactic reaction under continuous stimulation.

The figures shown in table 1 regarding the effect of size upon the survival show that the larger the animal the longer is the survival-time in potassium cyanide. These results are clear-cut in all respects save one. The rheotaxis both in percentage of responses and in efficiency of the reaction is practically identical, while the size differences and the survival-time differences are marked. But almost all the larger ones are male and most of the smaller ones are female and most of the records were taken during the breeding season. The females of the same age are usually smaller than the males, but not so much smaller as is here indicated. From general appearance these females were younger than the larger ones that were apparently the same age as the males.

An examination of the records of 58 males and 28 females from Stock III, mainly out of the breeding period, in which the size and rheotactic responses averaged approximately the same,

showed no definite difference in the survival-time of the two sexes. If anything, the females had the longer survival-time. This would tend to rule out the question of sex in the cases above noted but leaves that of the effect of the breeding season unanswered. The results may fairly be interpreted as showing a difference in survival-time due mainly to difference in age and perhaps partially to a differential action of breeding responses on males and females.

Very small isopods (1.5–1.75 mm. long) have a much reduced survival-time. Ten of this size lived only three hours and forty minutes in 0.0002 molecular potassium cyanide solution, while the mean for 72 adults averaging 10.7 mm. long, was five hours and fifty-nine minutes. This marked difference may have been partially due to the more tender external covering of the smaller isopods which allowed more direct action of the cyanide, but since the facts now at hand indicate that recently molted isopods live a longer, not a shorter, time than their mates, this does not seem sufficient ground to account for so great difference.

Finally, the most crucial evidence that resistance to cyanide does measure metabolism in isopods, is to be found in a comparison between the carbon dioxide output and the survival-time. A number of determinations of this character were made jointly with Dr. Shiro Tashiro at Woods Hole during the past summer (cf. Tashiro '13 p. 141). The results of these experiments will be published in detail elsewhere (Allee and Tashiro '14). Suffice it to say that in the twelve trials made, with one easily-explained exception, the resistance to potassium cyanide was inversely proportional to the rate of carbon dioxide production. As the experiments were run, this is the most delicate and accurate test and it is the more interesting in that the results with this method were quantitative.

In summing up the inquiry as to whether the resistance to relatively strong solutions of cyanide does measure the metabolic conditions of isopods, it is found that the survival-time is decreased by raising the temperature and by stimulation by shaking; that it is lengthened by decreasing the temperature; that larger (older) isopods have a longer survival-time than small (young) ones; and that the carbon dioxide production is inversely proportional to



the resistance to cyanide. From this evidence it seems safe to conclude that the cyanide resistance method does measure the metabolic state of isopods.

# RELATION BETWEEN RHEOTAXIS AND SURVIVAL-TIME IN POTASSIUM CYANIDE

## *The direct method*

From preceding work on this subject (Allee '12, '13) one would expect that the isopods giving the most highly positive rheotactic reaction, other things being equal, would die sooner in potassium cyanide than any others; that those giving a highly negative response would die next; that those with a high percentage of indefinite reactions would have a still longer survival-time; and that isopods that failed to respond to the current at all would live the longest. The results of experimental tests on the subject are listed in table 2.

The results from 150 individuals exhibited in table 2, in which a high percentage of positive reactions are contrasted with a low

TABLE 2

*Showing the relation between survival-time in potassium cyanide and the different rheotactic responses. Temperature 17 to 21° C.*

STOCK	RHEOTACTIC RESPONSES COMPARED	MOLECULAR STRENGTH OF KCN SOLUTION	NUMBER ISOPDS USED				AVERAGE RHEO- TACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS	AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	AVERAGE SURVIVAL- TIME IN HOURS AND MINUTES
			+	-	8	0				
I-II	60 per cent or more +	0.0002	41	88	7	5	2.64	10.2	5.24	
	60 per cent or more -	0.0002	19	13	82	5	2.63	10.2	6.22	
	40 per cent or less +	0.0002	25	16	63	21	2.54	10.4	6.39	
III	60 per cent or more +	0.0002	26	83	9	8	2.34	7.4	21.16	
	60 per cent or more -	0.0002	17	6	88	6	2.21	6.8	22.33	
	40 per cent or less +	0.0002	23	15	71	14	2.12	6.8	23.31	
III	60 per cent or more +	0.001	26	83	12	3	2.33	6.2	4.34	
	60 per cent or more -	0.001	16	5	91	4	2.24	5.9	5.15	
	40 per cent or less α	0.001	24	9	68	17	1.98	6.0	5.34	
	60 per cent or more 0	0.001	14	0	0	17	83	0.16	6.4	3.00

percentage, show in each case that the lower rate of positive rheotaxis accompanies a relatively long survival-time in the cyanide. That is, the isopods giving a low positive rheotactic reaction have on the average a slower rate of metabolism than do animals giving a higher percentage of positive responses. We are dealing here with average results only; individual results will be considered later (p. 410).

The position in the metabolic gradient of isopods that give a high percentage of negative reactions has been discussed in an earlier paper (Allee '13). From the evidence then at hand it was decided that under these conditions isopods occupied a place midway between that which accompanies a high degree of positiveness and a high degree of indefiniteness. That this conclusion is true is indicated by the results given in table 2, where averaged responses of 52 individuals from three sets of experiments have a survival-time intermediate between that given with a high percentage and that with a low percentage of positive responses.

In all the individuals tested in these experiments the negative rather than the indefinite response increased as the positive response lessened. For this reason there were too few instances where the isopods gave a majority of indefinite reactions to make their inclusion in the table of any value. However, the position upon the metabolic gradient of animals giving a high percentage of indefinite responses is firmly established by the earlier experiments (Allee '12, '13). These clearly show that the indefinite reaction accompanies a rate of metabolism lower than either of the other responses.

The survival-time of animals showing a high percentage of inaction in the current varies greatly. At times the survival-time is much longer than when movement occurs but on the average it is shorter than that of highly positive isopods. This is not surprising if one remembers that there is no rheotactic method for measuring the metabolic activity of the isopods that give no reaction to the current. They may be just lower than the lowest rate that would result in an indefinite reaction, or they may not be strong enough to turn over on their feet after the current has turned them on their backs. It is the first type that gives the

long survival-period while the very weak ones die soon after being placed in the cyanide. In this regard the cyanide method would fail to measure the metabolic rate. This may be due to there being just enough oxidation carried on to keep the isopod alive, so that when the cyanide decreases this ever so little death speedily results.

In another paper (Allee '13) it was pointed out that the efficiency of reaction in the water current varied more or less closely with the percentage of positive responses. That this is also an expression of the metabolic condition of the isopod is shown by the data presented in table 3.

TABLE 3

*Showing the relation between the survival-time in potassium cyanide and the efficiency of the response to the current of water. Temperature 17 to 21° C.*

STOCK	EFFICIENCY OF RESPONSES COMPARED	MOLECULAR STRENGTH OF KCN SOLUTION	NUMBER ISOPODS USED				AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	AVERAGE SURVIVAL- TIME IN HOURS AND MINUTES
			+	-	AVERAGE R.H.E.O- TACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS				
I-II	2.8 or more	0.0002	27	71	26	3	3.1	9.6	6.21
	2.0 or less	0.0002	13	43	18	36	1.7	11.4	7.14
	2.6 or more	0.0002	5	60	40		2.9	8.9	14.46
III	2.0 or less	0.0002	10	32	21	39	1.7	6.3	19.49
	2.6 or more	0.001	10	63	34	3	2.8	7.0	4.59
	2.0 or less	0.001	19	35	38	22	1.78	5.9	5.11

The results exhibited in table 3 are too few to be more than indicative; however, since they agree with all the other observations I think they approximate the truth of the situation. This is that while high efficiency in the current usually accompanies a larger percentage of positive responses and a higher rate of metabolism than low efficiency, yet considered alone the differences are neither so marked nor so sure as when the sign of the rheotactic reaction is made the index of the metabolic condition.

One pertinent comparison remains in the relation between survival-time and rheotaxis. What are the rheotactic reactions

which accompany relatively long and relatively short resistance to potassium cyanide? A synopsis of these results is presented in table 4.

Upon making this comparison one is struck, first by the relative closeness of the rheotactic responses given under the two conditions and second by the fact that the longer survival-times have always the lower percentage of positive responses. One of the factors causing the relatively low percentage of positive rheo-

TABLE 4

*Showing the relation between long and short survival-time and rheotaxis. Temperature 17 to 21° C.*

STOCK	SURVIVAL-TIMES COMPARED	NUMBER ISOPODS USED	AVERAGE SURVIVAL-TIME IN HOURS AND MINUTES				AVERAGE RHEOTACTIC REACTION IN PERCENTAGE OF TOTAL THIALS				AVERAGE EFFICIENCY		MOLECULAR STRENGTH OF KCN SOLUTION
			+	-	±	0	+	-	±	0	AVERAGE	LENGTH IN MM.	
I-II	5 hours or less.....	29	4.03	65	25	10	2.5	10.5	0.0002				
	7 hours or more.....	20	9.16	80	32	18	2.5	11.1	0.0002				
	18 hours or more.....	21	9.09	56	32	10	22.3	7.3	0.0002				
III	28 hours or more.....	18	39.10	47	41	9	32.1	6.5	0.0002				
	4 hours 15 minutes or less..	20	3.01	51	38	8	32.1	6.1	0.001				
	6 hours 15 minutes or more	16	8.58	34	43	18	52.1	6.1	0.001				

tactic reactions with the shorter survival-periods is that isopods sometimes give a high degree of negativeness with a relatively short survival-time (cf. Allee '13), although on the average such isopods live longer in the cyanide than do highly positive ones (see also p. 405).

#### *The indirect method.*

Only isopods from Stock II were tested by this method. These were placed in 0.00001 molecular potassium cyanide solution in two-liter flasks immediately after having been tested for rheotaxis. Three easily distinguished individuals were placed in each flask. In order to secure more even temperature the flasks were placed in running water. The temperature averaged 18° C., but

varied  $\pm 4^\circ$ . The isopods were examined each morning and night. As soon as one died it was removed with a long pipette. In general the method is, I believe, a trifle more reliable than the direct method since the exact determination of the death point is of less significance. However, since the method is so much slower it was used only to check the results obtained with the direct method. The findings are summarized in table 5. They support, as far as is possible with a limited number of cases, the findings with the direct method.

TABLE 5

*Showing the relation between survival-time in 0.00001 molecular potassium cyanide solution and the rheotactic reaction in Stock II. Temperature range 14 to 22° C.; average 18°*

RHEOTACTIC RESPONSES COMPARED	NUMBER ISOPODS USED	AVERAGE RHEOTACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS				AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	AVERAGE SURVIVAL-TIME IN DAYS
		+	-	x	0			
60 per cent or more +.....	19	86	5	9		2.7	9.6	5.7
40 per cent or less +.....	12	16	61	20	3	2.4	10.0	2.5

## DISCUSSION

It was shown in an earlier paper (Allee '12) that isopods of the pond mores are less positive in their rheotactic reaction than those of the stream mores. This is again illustrated by these studies and light is thrown on the metabolic condition of the isopods giving this difference in response. Thus the rheotactic reactions of 72 isopods of the stream mores (Stocks I and II), taken partially during the lowered responses of the breeding season, averaged 61 per cent positive, 28 per cent negative, and 11 per cent indefinite, with an average efficiency in the current of 2.5. These gave a mean survival-time in 0.0002 molecular potassium cyanide of five hours and fifty-nine minutes.

Of the pond mores 51 were killed in 0.0002 molecular cyanide solution with an average survival-time of twenty-three hours and

ten minutes. These pond isopods had given an average rheotactic response of 51 per cent positive, 37 per cent negative, 10 per cent indefinite and 2 per cent no reaction. Their average efficiency in the current was 2.2. Another lot of 55 pond isopods which gave a rheotactic average similar to the above, were killed off from normal conditions in 0.001 molecular potassium cyanide; these gave a mean resistance of five hours and fourteen minutes, just forty-five minutes less than that given by the stream isopods to a cyanide solution one-fifth as strong.

All this means that the stream isopods, the ones that as a rule are more highly positive to and more efficient in their reactions in a water current than pond isopods, have a shorter survival-time in equimolecular solutions of potassium cyanide and therefore have a higher rate of metabolism than do the pond isopods.

If only the highly positive isopods of the two mores are chosen for a comparison of their resistance to potassium cyanide, another situation is revealed. The individuals of the two groups selected on the basis of the similarity of their rheotactic response show a great difference in their survival-time in equimolecular solutions of the cyanide and hence in their metabolic rate. In table 2 (p. 404) the stream isopods with a rheotactic reaction 60 per cent or more positive averaged 88 per cent positive and gave a mean survival-time in 0.0002 molecular potassium cyanide of five hours and twenty-four minutes. Isopods from the pond mores selected on the same basis averaged 83 per cent positive and gave a survival-time of twenty-one hours and sixteen minutes, in the same strength of cyanide solution. Here is a great difference in the metabolic activity of the two groups of isopods but a close similarity in their rheotactic reaction. It can also be seen in table 2 that the pond isopods that give a low positive response have a longer survival-time in potassium cyanide than those that are more highly positive. This must mean that the rate of metabolism which is accompanied by a high degree of positiveness is relatively high when compared with the mean metabolic rate given under the conditions to which the isopods are acclimated, but that it may not be an absolutely high rate when compared

with a fixed standard. This topic will immediately be discussed more fully in dealing with the reactions of individual isopods.

Thus far I have been dealing with averaged results. For generalizations these are necessary and in order to save space the individual records have not been given. How far does the work with individuals support the averaged results? Before taking up this point it is necessary to point out that each individual has in all probability a rate of metabolic processes slightly different from that of any other isopod and further, that it is not a fixed standard rate of metabolism that finds its expression in a high rate of positiveness but rather a relative rate. That is, when the metabolism of an isopod is rapid for that individual it tends to go positive to the water current, when less rapid, negative, when still less rapid, indefinite, and when least rapid no reaction at all is given. But a rate of metabolism that is rapid for one isopod may be slow for another, and intermediate for a third. Now all three would show the same survival-time in potassium cyanide and yet their rheotactic reaction would be highly different. Thus for example, isopods 96 and 101 from Stock III had a survival-time of three hours and forty-five minutes in 0.001 molecular cyanide solution. The former gave 50 per cent positive, the latter 100 per cent positive reactions. Isopod No. 97 was killed in the same solution with the other two with a survival-time of three hours and fifteen minutes, yet its rheotactic reaction was only 60 per cent positive. Since it is obviously impossible to test the survival-time of an isopod in its varying rates of metabolism, one is forced to do the next best thing, that is, take average results.

There is still another complication in this regard. An individual isopod may give identical rheotactic reactions when its rate of metabolism varies greatly. Thus an isopod kept at 20°C. comes to have a normal mean metabolic rate, also a normal mean rheotactic response. When the metabolic rates goes above this normal mean the isopod tends to become more positive, when below, less positive to water currents. Put the same isopod at 5°C. The metabolic rate is depressed and the positiveness also decreases. But in time the isopod becomes acclimated; a new metabolic mean is established and the rheotactic reaction goes up

to about its old average and plays up and down as the metabolic rate varies about its new mean. So the rheotactic reaction is an expression, not of the absolute metabolic rate of the animal, but of the relative metabolic conditions under which the isopod is acclimated for the time being. Further experimental evidence on this point will be published elsewhere in conjunction with Dr. Shiro Tashiro.

But even with this relative scale of metabolism determining the rheotactic reaction of the isopod in place of an absolute scale, the fact remains that individuals having a short survival-time usually have a high rate of positiveness to a water current. This was determined for individuals as follows: An assistant read me the rheotactic reaction of isopods in groups of two from the same stock, giving individuals that had at least 10 per cent of difference in their positive rheotactic response. Basing judgment on the rheotactic reaction alone I was able to predict the relative survival-time in 106 out of the 160 cases tried. This shows that the rheotactic reaction in 66 per cent of the cases was a correct indicator of the absolute metabolic conditions of the isopods compared. Isopods with a highly negative reaction gave the most difficulty, while it was impossible to predict the survival-time of isopods that did not move at all in the current. The explanation of this difficulty has already been given (pp. 405-406).

Jennings ('06) clearly outlined the problems in this particular field of animal behavior when he said, "We are compelled to assume the existence of changing physiological states. This assumption besides being logically necessary, is of course supported by much positive evidence drawn from diverse fields, and there is reason to believe that in time we shall be able to study these states directly."

With the application of the cyanide method to problems of animal behavior this prophecy is a step nearer its final fulfilment and it is now possible to demonstrate directly as regards the rheotactic reaction of isopods that high positiveness is the expression of a relatively high rate of metabolism, and low positiveness, of a low metabolic rate.



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# STUDIES ON THE DYNAMICS OF MORPHOGENESIS AND INHERITANCE IN EXPERIMENTAL REPRODUCTION

## VII. THE STIMULATION OF PIECES BY SECTION IN PLANARIA DOROTOCEPHALA

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FOUR FIGURES

### I. METHODS

By means of the direct susceptibility method it is possible to follow the changes in rate of metabolism resulting from section in pieces. This method, which has been described in detail elsewhere (Child '13 a), consists in determining the susceptibility of individuals or pieces to concentrations of KCN, alcohol, and so forth, which kill within a few hours. In such concentrations the higher the general rate of metabolism the greater the susceptibility and the shorter the life of the animal or piece in the solution. In this way the susceptibility serves as a means of distinguishing and comparing the general rate of metabolism in different individuals, regions, or pieces. Since the changes in susceptibility following section depend upon size of piece and region of the body from which it is taken it has been found most satisfactory to determine the susceptibility of series of pieces, representing both different fractions of the body length and different regions of the body, at various intervals after section. Of course a different series of pieces must be used for each susceptibility determination but by standardizing the material used and the external conditions a remarkable uniformity of results is possible.

The method of experiment is as follows: The bodies, exclusive of the heads, of ten worms of equal length and similar physiologi-

cal condition and always from the same stock, are cut into fourths, sixths, eighths, or twelfths; each lot of ten pieces representing approximately the same region of the body is kept together and isolated from other lots and its susceptibility tested at the desired time after section.

In all the susceptibility determinations of pieces presented below animals from the same stock were used and great care was exercised in selecting those of equal size, as experience has shown that in a particular stock size is the best criterion of physiological condition, and also in cutting the pieces so that they should represent as nearly as possible the same regions of the bodies of different individuals. It is of course impossible to avoid some differences in size and level of pieces but the results will show how unimportant these differences are. In all susceptibility determinations KCN 0.001 *m* was used in filtered water from the same source and at the same temperature and the same quantity of KCN solution was used in each case.

In this manner the susceptibility of series of ten each of fourths, sixths, and eighths, of wellfed animals 18 mm. in length has been determined, immediately, twelve, twenty-four, forty-eight, seventy-two, ninety-six and one hundred and twenty hours after section, and for some series of pieces three and six hours after section also. For twelfths the susceptibility has been determined, immediately, twenty-four and forty-eight hours after section, but it is impossible to avoid considerable relative differences in size in pieces as small as these and such differences complicate the results. With pieces smaller than twelfths no general susceptibility determinations have been attempted.

"Immediately after section" in these series means that as soon as the cutting of the series of pieces was completed they were placed in KCN. Since the preparation of the series requires from ten minutes to an hour according to the size and number of pieces the first pieces cut remain in water for some time before being placed in KCN. It was ascertained, however, that this made no difference in the results since the susceptibility does not change appreciably during several hours after section. In nearly

all cases the determination for each size and each time has been made repeatedly so that conclusions do not rest on single series and besides this the results of these experiments are amply confirmed by large numbers of others in which only certain regions of the body instead of the whole were used in susceptibility determinations in relation to size and region.

In the susceptibility determinations, as stated the whole body except the head was cut into fourths, sixths, etc., and the susceptibility of all pieces was determined and recorded, but in the following tables only the pieces from the first zoöid (see Child '11 c) are included. The pieces of the posterior zoöids show nothing fundamentally different from those of the first zoöids and double the length of the tables. Only a sufficient number of susceptibility records are given to show the general course of the changes in susceptibility. The tables of these records given below are supplemented and confirmed by many other similar series in my notes.

The tables are in the form of the susceptibility tables in an earlier paper (Child '13 a). The column headed "length of time" gives in hours and minutes the length of time that the animals or pieces have been in the solution at each observation. The column headed "lots" gives the serial numbers for each time of the different lots of ten each, beginning with the most anterior pieces as Lot 1. The lot-numbers are the same as the numbers of the pieces in the corresponding figures.

The five columns headed "I to V" under "stages of disintegration" distinguish more or less arbitrarily five stages in the visible changes which the animals undergo in dying. These five stages are briefly as follows:

I. Still intact, showing no appreciable disintegration. Such animals or pieces are always alive and moving about.

II. In whole animals, from the first traces of disintegration to the beginning of disintegration along the margins of the body, which usually follows disintegration of the head. In pieces from the beginning of disintegration at one or both ends to beginning of disintegration along the lateral margins. Considerable motor activity may still be present.

III. In both whole animals and pieces disintegrations of lateral margins of body until it is completed and swelling of body begins. Movement may still occur to some extent in the intact parts.

IV. Swelling of the body to complete loss of epithelium and loss of shape. Motor activity has ceased.

V. All further changes after swelling and loss of shape are completed.

The recognition of these different stages makes it possible to distinguish more clearly slight differences in susceptibility which would otherwise not be evident.

The numbers in each of the five columns for each time and each lot are the actual numbers of animals or pieces of that lot which are in that stage at that time. Lots of ten each were used and condition was recorded every half-hour in all cases.

From these tables it is possible to determine at a glance the regional differences in susceptibility and by comparison of the tables for pieces of different size the effect of size and region upon the changes in susceptibility following section appears and comparison of the susceptibilities of pieces of the same size at different times shows the course of the susceptibility changes.

## II. STIMULATION

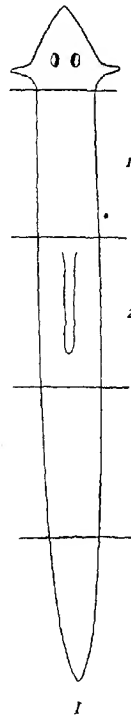
### *1. The susceptibility of whole animals*

In order to show how the susceptibility of the pieces changes following section it is necessary to determine the susceptibility of whole animals as a basis for comparison. Table 1 gives the susceptibility of a lot of ten worms 18 mm. in length, that is, of the same size and physiological condition as the animals used for the preparation of pieces.

### *2. The one-fourth pieces*

The regions of the body represented are indicated in figure 1. Pieces 1 and 2 represent the body of the first zoöid and only the records of these pieces are included in the tables.

Comparison of table 2, the one-fourth pieces, immediately after section, with table 1, the whole worms, shows that the susceptibility of the pieces is somewhat greater than that of the whole animals. Susceptibility determinations twelve and twenty-



four hours after section show a lower susceptibility than table 2 but no great change occurs during this period. Table 3, forty-eight hours after section, shows a susceptibility lower than that of whole animals.

TABLE 1

*Susceptibility of ten worms 18 mm. in length to KCN 0.001 m*

LENGTH OF TIME IN KCN	STAGES OF DISINTEGRATION				
	I	II	III	IV	V
4.00	4	6			
4.30	4	5	1		
5.00		7	3		
5.30		3	7		
6.00			10		
6.30			10		
7.00			7	3	
7.30			4	4	2
8.00				4	6
8.30				2	8
9.00					10

TABLE 2

*Susceptibility to KCN 0.001 m of one-fourth pieces immediately after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.30	1	7	3			
	2	9	1			
4.00	1		9	1		
	2	2	8			
4.30	1		6	3	1	
	2		7	3		
5.00	1			7	3	
	2		2	7	1	
5.30	1			7	3	
	2			9	1	
6.00	1			2	7	1
	2			6	4	
6.30	1				7	3
	2				9	1
7.00	1				4	6
	2				5	5
7.30	1					-10
	2				1	9
8.00	2					10

TABLE 3

*Susceptibility to KCN 0.001 m of one-fourth pieces forty-eight hours after section*

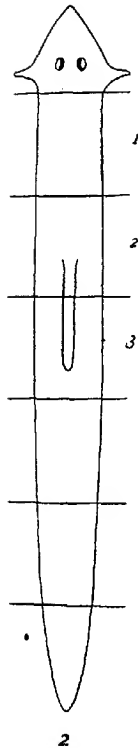
LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
5.30	1	9	1			
	2	10				
6.00	1	4	6			
	2	8	2			
6.30	1	1	9			
	2	8	2			
7.00	1	1	5	2	2	
	2	6	2			
7.30	1	1		5	2	2
	2	2	3	3	2	
8.00	1		1	2	2	5
	2	2	3	3	1	1
8.30	1			1	2	7
	2		2	3	3	2
9.00	1			1	1	8
	2		2	1	3	4
9.30	1				1	9
	2			2	3	5
10.00	1					10
10.30	2				2	8
11.00	2					10

TABLE 4

*Susceptibility to KCN 0.001 m of one-fourth pieces one hundred and twenty hours after section.*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.00	1	7	3			
	2	4	6			
3.30	1		9	1		
	2		4	6		
4.00	1		5	4		1
	2		4	4	2	
4.30	1		4	4		2
	2		4	3	3	
5.00	1		4	1	3	2
	2			4	4	2
5.30	1		2	2	1	5
	2				5	5
6.00	1			2	1	7
	2					10
6.30	1				3	7
7.00	1					10





From forty-eight hours on, the susceptibility begins again to increase as the pieces undergo reconstitution. At seventy-two hours it is somewhat greater, at ninety-six hours still greater and table 4 for one hundred and twenty hours shows that it has now again become greater than that of the whole worms (cf. table 1).

As regards the susceptibility of the first and second fourths, tables 2 to 4 show that the first fourth is at all times more susceptible than the second, that is, the original axial gradient (Child 12 a, 13 b, 13 c) appears in the difference in susceptibility between the first and second fourths.

3. *The one-sixth pieces*

Figure 2 shows the region of body included in each of the one-sixth pieces. In table 5 to 8 only records of the pieces 1, 2 and 3 which represent the body of the first zoöid are included.

Comparison of tables 5 to 8 with each other and with table 1 shows that the susceptibility of the one-sixth pieces immediately after section (table 5) is greater, but after twelve hours (table 6) is less than that of whole worms.

TABLE 5

*Susceptibility to KCN 0.001 m of one-sixth pieces immediately after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION			
		I	II	III	IV
3.30	1	10			
	2	7	3		
	3	7	3		
4.00	1	7	3		
	2	4	6		
	3	4	6		
4.30	1	2	8		
	2	1	6	3	
	3	2	6	2	
5.00	1	2	6	1	1
	2		2	6	2
	3		4	5	1
5.30	1	2		7	1
	2			6	3
	3		1	7	1
6.00	1		2	4	1
	2			2	6
	3			3	2
6.30	1		2	1	2
	2				5
	3			1	3
7.00	1			2	1
	2				4
	3				1
7.30	1				1
	2				
	3				
8.00	1				

Susceptibility determinations twenty-four and forty-eight hours (table 7) after section show a slight further decrease in susceptibility. From this time on the susceptibility begins to increase as reconstitution proceeds. At seventy-two hours it has increased slightly, at ninety-six still more and at one hundred and twenty hours it is once more higher than that of the whole worm. As reconstitution goes on it continues to increase.

These sixths show another interesting feature. Comparison of Lots 1, 2 and 3 in table 5 shows that 2 and 3 are considerably

TABLE 6  
*Susceptibility to KCN 0.001 m of one-sixth pieces twelve hours after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
5.00	1	9	1			
	2	10				
	3	8	2			
5.30	1	7	3			
	2	10				
	3	7	2	1		
6.00	1	5	4	1		
	2	8	2			
	3	6	2	2		
6.30	1	4	3	2	1	
	2	5	3	2		
	3	3	1	3	2	1
7.00	1		4	3	2	1
	2	5		3	2	
	3	1	2		4	3
7.30	1			4	1	5
	2	4	1	1	2	2
	3		2	1	1	6
8.00	1			1		9
	2	1	2	2		5
	3		1		2	7
8.30	1				1	9
	2		1	2	1	6
	3			1		9
9.00	1					10
	2			2		8
	3					10
9.30	2				2	8
10.00	2					10

more susceptible than 1; in other words, the susceptibility of posterior is greater than of anterior pieces. This change constitutes, so far as the different pieces are concerned, a reversal of the axial gradient of the first zoöid of uninjured worms. It is a characteristic feature not only of sixths but, as will appear below, of all shorter pieces and is due to the fact that posterior pieces are more stimulated by the act of section than anterior.

After twelve hours (table 6) the effect of this stimulation has disappeared in Lot 2 so that its susceptibility is lower than that

TABLE 7

*Susceptibility to KCN 0.001 m. of one-sixth pieces forty-eight hours after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
6.00	1	9	1			
	2	10				
	3	10				
6.30	1	7	2			
	2	10				
	3	10				
7.00	1	4	4	2		
	2	10				
	3	10				
7.30	1	2	2	3	3	
	2	9		1		
	3	9	1			
8.00	1		2	1	2	5
	2	5	3	1	1	
	3	4	3	2	1	
8.30	1			2		8
	2	1	6	1	1	1
	3	2	4	1	2	1
9.00	1				2	8
	2	1	3	1	5	
	3	2	1	1	3	3
9.30	1					10
	2	1			6	3
	3		1		3	6
10.00	2	1				9
	3				1	9
10.30	2					10
	3					10

of Lot 1 while Lot 3 has not yet fully recovered and its susceptibility is about the same as that of Lot 1.

After twenty-four hours further recovery has occurred and after forty-eight hours (table 7) the susceptibility of Lot 2 is lower than that of Lot 1 and that of Lot 3 slightly lower than that of Lot 2, that is, the original axial gradient of the whole worm has now reappeared in the relative susceptibilities of the pieces.

As reconstitution proceeds, the susceptibility of all the pieces rises and since the more posterior regions of the first zoöid undergo more reorganization than the anterior regions in giving rise to a new individual, the susceptibility of the posterior pieces rises

TABLE 8

*Susceptibility to KCN 0.001 m. of one-sixth pieces one hundred and twenty hours after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.00	1	4	6			
	2	6	4			
	3	9	1			
3.30	1		10			
	2		10			
	3	3	7			
4.00	1		9	1		
	2		10			
	3	3	7			
4.30	1		4	6		
	2		7	3		
	3		7	3		
5.00	1		4	3	2	1
	2		5	3	2	
	3		4	5	1	
5.30	1			5		5
	2			6	1	3
	3			6	3	1
6.00	1				2	8
	2				4	6
	3			2	2	6
6.30	1					10
	2					10
	3				2	8
7.00	3					10

more than that of the anterior. In table 8, one hundred and twenty hours after section, this has occurred and the susceptibility of all three lots is almost the same. Still later the susceptibility of the posterior pieces provided they undergo complete reconstitution, may become even greater than that of the anterior pieces.

#### *4. The one-eighth pieces*

The regions represented by the pieces are shown in figure 3. In tables 9 to 12, only the records of pieces 1 to 4 are given.

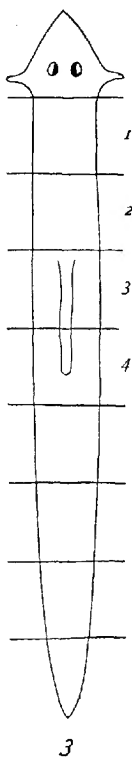
Table 9 compared with tables 5, 2 and 1 shows that in the first four one-eighth pieces the susceptibility is higher immediately after section than in the one-sixth pieces or one-fourth pieces or in the whole worms. Not only do these pieces complete disintegration earlier than the others, but the first traces of disintegration appear earlier.

After twelve hours, however, as shown in table 10, the susceptibility has decreased so that it is about the same as that of the whole animals. At twenty-four hours and also at forty-eight hours (table 11) it is still about the same. Seventy-two hours after section the susceptibility is again higher, at ninety-six hours still higher and at one hundred and twenty hours (table 12) it is higher still. Many of these short pieces in the more posterior regions do not undergo complete reconstitution but remain headless and in these the susceptibility does not undergo as great an increase as in those which produce wholes.

But the one-eighth like the one-sixth pieces show regional differences in the increase in susceptibility immediately following section. In table 9 it is evident that the anterior pieces (Lot 1) are in general least susceptible, the second eighths (Lot 2) considerably more susceptible, the third eighths (Lot 3) slightly more susceptible than the second, and the fourth eighth (Lot 4) about like the third. Here, as in the one-sixth pieces, the regions of the body which in the uninjured animal have lower susceptibility show a higher susceptibility after section, that is, the stimulation resulting from section is least in the anterior piece and increases

from piece to piece in the posterior direction except in the last two pieces where there is little difference.

After twelve hours (table 10) this stimulation has disappeared and now the susceptibility decreases from piece to piece in the posterior direction, i e., the original axial gradient has reappeared.



At twenty-four hours the relative susceptibilities of the one-eighth pieces remain about the same as at twelve hours. In table 11 (forty-eight hours) some slight irregularities in the susceptibility of different pieces appear. Lot 1 is most susceptible, Lot 2 less

TABLE 9

*Susceptibility to KCN 0.001 m. of one-eighth pieces immediately after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION			
		I	II	III	IV
2.00	1	10			
	2	6	4		
	3	7	3		
	4	10			
2.30	1	10			
	2	3	7		
	3	4	5	1	
	4	6	4		
3.00	1	7	3		
	2	2	5	3	
	3	4	5	1	
	4	4	5	1	
3.30	1	6	4		
	2		8	2	
	3		5	4	1
	4		6	4	
4.00	1	3	7		
	2		6	3	1
	3		4	2	2
	4		4	2	2
4.30	1		6	4	
	2		1	4	4
	3		2	3	2
	4			3	5
5.00	1			9	1
	2			3	2
	3			1	4
	4			2	1
5.30	1			6	4
	2				3
	3				2
	4				2
6.00	1			1	9
	2				
	3				
6.30	1				5
	2				5
	3				5
7.00	1				10



TABLE 10

*Susceptibility to KCN 0.001 m. of one-eighth pieces twelve hours after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
4.00	1	9	1			
	2	10				
	3	8	2			
	4	10				
4.30	1	8	1	1		
	2	9	1			
	3	8	2			
	4	9	1			
5.00	1	4	3	2	1	
	2	5	3	2		
	3	5	2	3		
	4	8	1	1		
5.30	1	1	4	3	2	
	2	4	2	3	1	
	3	5	1	2	2	
	4	6	2	2		
6.00	1	1	1	5	2	1
	2	3	1	2	3	1
	3	3	1	3	2	1
	4	4	2	2	2	
6.30	1	1	1	1	4	5
	2	2	2		4	2
	3		2	2	4	2
	4	3	1	1	2	3
7.00	1			1		9
	2	1		3	1	5
	3		1	1	3	5
	4	1		1	5	3
7.30	1					10
	2		1	1	1	7
	3				2	8
	4	1		2		7
8.00	2		1			9
	3					10
	4	1				9
	5					9
8.30	2			1		9
	4			1		9
9.00	2				1	9
	4				1	9
9.30	2					10
	4					10

TABLE 11  
Susceptibility to KCN 0.001 m. of one-eighth pieces forty-eight hours after section

LENGTH OF TIME IN KCN	Lots	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.30	1	9	1			
	2	10				
	3	10				
	4	10				
4.00	1	7	3			
	2	10				
	3	10				
	4	10				
4.30	1	6	4			
	2	10				
	3	9	1			
	4	10				
5.00	1	4	5	1		
	2	10				
	3	9	1			
	4	10				
5.30	1		7	3		
	2	7	3			
	3	5	4	1		
	4	9	1			
6.00	1		4	3	3	
	2	4	6			
	3	4	2	3	1	
	4	8	2			
6.30	1		2	3	4	1
	2	4	3	3		
	3	1		5	3	1
	4	2	8			
7.00	1			3	2	5
	2	1	6		3	
	3		1	2	2	5
	4	1	6	1	2	
7.30	1			1	3	6
	2	1	3	3		3
	3			2	3	5
	4		3	4	2	1
8.00	1					10
	2		1	3		6
	3				1	9
	4		1	1	2	6
8.30	2			1	1	8
	3				1	9
	4				1	9
	2				1	9
9.00	3				1	9
	4					10
9.30	2					10
	3					10

susceptible, Lot 3 more susceptible than Lot 2, and Lot 4 again less susceptible than Lot 3. These irregularities are undoubtedly incidental and due either to differences in size of pieces, too large pieces showing too low and too small too high a susceptibility, or to stimulation of some of the pieces which cannot always be avoided and which distinctly increases susceptibility.

TABLE 12

*Susceptibility to KCN 0.001 m of one-eighth pieces one hundred and twenty hours after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.30	1	7	3			
	2	8	2			
	3	9	1			
	4	8	2			
4.00	1	1	9			
	2	1	9			
	3	5	5			
	4	5	5			
4.30	1		5	5		
	2	2	4	4		
	3	4	4	1	1	
	4	4	4	1	1	
5.00	1			6	2	2
	2		2	2	2	4
	3	2	4	2		2
	4	3	4	1	1	1
5.30	1				6	4
	2			1	1	8
	3		3	3		4
	4		2	3		5
6.00	1					10
	2			1		9
	3			1	3	6
	4			3		7
6.30	2					10
	3				2	8
	4				3	7
7.00	3					10
	4					10

At seventy-two and ninety-six hours the gradient in susceptibility from piece to piece is essentially that of the uninjured animal. At one hundred and twenty hours the same is true, as table 12 shows. Here Lot 1 has the greatest susceptibility, Lot 2 is next, Lot 3 next, and Lot 4 about like Lot 3. One-eighth pieces from the posterior region of the first zoöid undergo but little reconstitution and almost never produce whole animals while in the more anterior pieces the frequency of complete reconstitution is greater, consequently the differences in susceptibility in table 12 one hundred and twenty hours after section are due in part to a greater degree of reconstitution in anterior than in posterior pieces.

#### *5. The one-twelfth pieces*

The difficulty of cutting even large individuals into as many as twelve pieces of anything like equal size sets a limit to the study of consecutive series of pieces. It is of course possible to cut single pieces much smaller than this with a considerable degree of accuracy but in consecutive series the difficulties are much greater.

Figure 4 indicates the regions represented by the first six pieces which make up the first zoöid. Tables 13 and 14 give the susceptibilities immediately and forty-eight hours after section.

The susceptibility of all the pieces is very high immediately after section, being higher than that of any of the larger pieces (tables 2, 5, 9) or of the whole worms (table 1). The survival-time of even the least susceptible pieces is only half that of the whole worms and larger pieces. These short pieces are evidently more stimulated by the act of section than are larger pieces. After forty-eight hours, however, (table 14) the susceptibility of all pieces has decreased greatly and is only slightly higher than that of uninjured animals. The effect of section has at least largely disappeared.

Among the one-twelfth pieces only the more anterior produce whole animals and these do not in all cases. The more posterior pieces usually remain headless. Consequently the later increase

in rate associated with reconstitution is much greater in the anterior than in the posterior pieces. It is in general similar to that in the one-eighth pieces and requires no special comment.

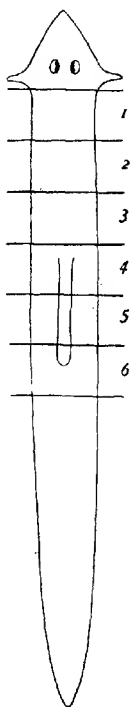


TABLE 13

*Susceptibility to KCN 0.001 M of one-twelfth pieces immediately after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
1.00	1	10				
	2	10				
	3	8	2			
	4	7	3			
	5	7	3			
	6	7	3			
1.30	1	10				
	2	8	2			
	3	6	4			
	4	6	3		1	
	5	2	6	2		
	6		7	1	2	
2.00	1	2	5	3		
	2		5	3	2	
	3		5	3	1	1
	4		3	3	2	2
	5		1	3	2	4
	6		1	1	4	4
2.30	1	1	1	4	3	1
	2			3	4	3
	3			3	2	5
	4			2	4	4
	5			2	1	7
	6			1		9
3.00	1		1	1		8
	2			1	1	8
	3			1	1	8
	4			1		9
	5				1	9
	6				1	9
4.00	1			1		9
	2					10
	3					10
	4					10
	5					10
	6					10
	1					10

TABLE 14

*Susceptibility to KCN 0.001 m of one-twelfth pieces forty-eight hours after section*

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.00	1	9		1		
	2	9				1
	3	9				1
	4	10				
	5	10				
	6	10				
3.30	1	9			1	
	2	7	2			1
	3	9				1
	4	10				
	5	10				
	6	10				
4.00	1	9				1
	2	7	1	1		1
	3	9				1
	4	10				
	5	9		1		
	6	10				
4.30	1	6	3			1
	2	4	3			3
	3	4	5			1
	4	6	4			
	5	8	1			1
	6	10				
5.00	1	2	4	3		1
	2	2		1	1	6
	3	3	4		2	1
	4	5	2	1	1	1
	5	8				2
	6	10				
5.30	1		2	2	2	4
	2		1	1		8
	3		2	4	3	1
	4	5				5
	5	7		1		2
	6	7	3			
6.00	1			4		6
	2		1			9
	3			1	2	7
	4	2	3			5
	5	6	1			3
	6	6	1	1	2	

TABLE 14—Continued

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
6.30	1				1	9
	2			1		9
	3				1	9
	4		3	2		5
	5	5	1			4
	6	3	3		2	2
7.00	1	—	—	—	—	10
	2	—	—	—	—	10
	3	—	—	—	—	10
	4			2	1	7
	5	2	1	2		5
	6		2	4		4
7.30	4				2	8
	5			1	2	7
	6				2	8
8.00	4	—	—	—	—	10
	5	—	—	—	—	10
	6	—	—	—	—	10

## III. CONCLUSIONS

A number of conclusions which will be shown later to be of the greatest importance for an understanding of the process of reconstitution are to be drawn from the data presented in the tables above. These are briefly stated and discussed in the following paragraphs.

1. *A temporary increase in rate of metabolism, a 'stimulation' lasting at least several hours, results from the act of section.*

Such a stimulation is to be expected for the act of section severs nerve cords and of course produces extensive injury to other tissue. Susceptibility determinations of pieces three hours after section, of which the records are not given, show that at that time the susceptibility is as high as immediately after section. From this time on it gradually decreases and twelve hours after section the susceptibility of the pieces is about the same as that of the uninjured animals.



This also is to be expected, for the piece is no longer in connection with its chief sources of stimulation, that is, first the head region and secondly other parts of the body. As regards movement it is much less active than the intact animal and if it were not for the wounds at its ends and the growth processes beginning there its rate of metabolism would undoubtedly fall far below that of the uninjured animal.

2. *The temporary increase in rate of metabolism following section varies in amount inversely as the length of the piece.*

A comparison of tables 2, 5, 9 and 13 will show that in general the smaller the pieces the higher the susceptibility. The one-fourth pieces show in the tables practically no increase in susceptibility following section. As a matter of fact the regions adjoining the cut ends do show an increased susceptibility but it does not involve the piece as a whole.

That the shorter piece should be more stimulated than the longer by section is also in accord with expectation. The shorter the piece the more direct the injury and resulting stimulation of its conducting paths and tissues. In very short pieces almost the whole length becomes involved in the wound reaction at the two ends. Tables 10 and 14 show that the susceptibility of the one-eighth and one-twelfth pieces does not fall during the forty-eight hours following section to so low a level as that of the longer pieces. This difference is undoubtedly due to the fact that in the shorter pieces the wound reaction affects the whole rather than merely the two terminal regions as in the longer pieces. In the long pieces the susceptibility of the two terminal regions is distinctly higher than that of the rest of the piece at this stage.

3. *The temporary increase in rate of metabolism following section varies in amount according to the region of the original body which the piece represents, being least in the most anterior and increasing in successively more posterior pieces.*

This regional difference in stimulation is associated with length of piece and is not apparent in the long one-fourth pieces, but in the one-sixth, one-eighth and one-twelfth pieces it is distinct. The rate of metabolism in the first zoöid of uninjured animals is highest in the anterior region and decreases more or less regularly

in the posterior direction (Child '13 c). In the one-fourth pieces of table 2 this gradient continues to exist after section, the rate of the second fourth being lower than that of the first. In some other series the rates of first and second fourths immediately after section are about the same, that is, the original gradient has disappeared. In the one-sixth, one-eighth and one-twelfth pieces (tables 5, 9 and 13) we see that in general the susceptibility increases from piece to piece posteriorly, i. e., the original axial gradient is reversed. This can be due only to the fact that more posterior pieces are more strongly stimulated by section and this is actually the case. A comparison of the susceptibilities of Lot 1, which represents the most anterior fourth, sixth, eighth or twelfth in tables 2, 5, 9 and 13, shows that the susceptibility of this anterior region is but little affected by length of piece and undergoes but little increase after section until we reach the one-twelfth pieces, where the increase is greater than in the longer pieces. The more posterior pieces are much more affected by section and the tables show that in general the more posterior the level of a piece the greater the degree of stimulation following section.

As regards the regional differences in rate immediately after section, the results of the susceptibility method have been confirmed by estimations of  $\text{CO}_2$  production very kindly made at my request by Dr. Tashiro with the apparatus devised by him (Tashiro '13). In one-fourth pieces or larger the second piece shows a lower rate of  $\text{CO}_2$  production than the anterior. In one-sixth pieces the anterior piece has the lowest rate of  $\text{CO}_2$  production and the rate increases posteriorly from piece to piece.

This regional difference in degree of stimulation agrees with the wellknown fact of observation that when such animals as earthworms and planarians are cut in two the posterior piece usually reacts much more strongly than the anterior and it is also in full accord with the theory of antero-posterior dominance developed in preceding papers (Child '11 d, '13 b, '13 c), in fact it constitutes valuable experimental evidence for this theory.

The greater stimulation of posterior as compared with anterior regions in consequence of section can only mean that the rate of

metabolism in the anterior region is to a greater degree independent of conditions in other parts of the body and so is but little altered when conducting paths are cut. The rate of metabolism in the posterior region on the other hand, must be in high degree dependent upon its connection with other regions, for when this connection is severed the stimulation of conducting paths increases its rate very greatly. The more anterior levels of the body are largely independent of the posterior while the more posterior levels are largely dependent upon the anterior. The degree of independence or subordination of a body-region then depends upon its level in the body.

There is thus an axial gradient in degree of stimulation resulting from section with its lowest point at the anterior and its highest point at the posterior end and this is evidently the expression of a gradient in degree of subordination.

That these gradients are expressions of the axial gradient in rate of metabolism described in the preceding paper (Child '13 a) cannot be doubted. It was pointed out in that paper that the region of highest metabolic rate in a living cell or cell aggregate must be much more independent of other regions than they are of it and so must inevitably dominate other parts to a greater or less extent and within a greater or less distance from it. In *Planaria* where a simple axial gradient in rate from the head posteriorly exists the head is unquestionably the dominant region. As regards functional relations this is self-evident but it is also true at least for certain features of morphogenesis (Child '11 d). As pointed out above, the stimulation gradient in *picces* is merely an expression under special conditions of the axial gradient and of antero-posterior dominance. The increase in stimulation from section with increasing distance from the anterior end means simply increasing dependence upon stimuli coming from more anterior regions.

4. *A remarkable relation between frequency of head formation and degree of stimulation after section exists. The greater the increase in rate of metabolism after section the less the frequency of head-formation.*

In the first paper of this series (Child '11 c) it was shown that the frequency of head-formation *decreases* as the length of the piece decreases and also from anterior to posterior regions. In the present paper we have seen that the degree of stimulation *increases* as the length of the piece decreases and also that the degree of stimulation is greater in posterior than in anterior pieces. It would seem that these two features, degree of stimulation after section and frequency of head formation, must be in some way associated or have a common foundation. But before the relation between them can be made clear it is necessary to find when in the history of a piece it is determined whether the piece shall give rise to a head or not. And consideration of this problem, the time of head determination, must be postponed to another paper. The chief purpose of the present paper, however, is to point out this inverse relation between head frequency and degree of stimulation after section. The following paper will afford an insight into the nature of the relation.

*5. After the temporary increase in rate of metabolism following section has disappeared a second slow and (relatively) permanent increase in rate occurs, in connection with reconstitution and this is a process of rejuvenescence resulting from the reorganization and reduction of the pieces.*

The writer has called attention elsewhere (Child '11 a, '13 d) to the occurrence of rejuvenescence in connection with experimental and asexual reproduction in planarians. The susceptibility tables in the present paper show merely the earliest stages of this process at one hundred and twenty hours after section (tables 4, 8, 12). This slow increase in rate in the pieces, which begins after three or four days and which indicates the beginning of rejuvenescence is relatively permanent, i. e., it disappears only gradually as the animal undergoes growth and becomes older in consequence of feeding. The degree of rejuvenescence is in general inversely proportional to the size of the piece and directly proportional to the degree of reconstitucional change. In this connection another expression of the axial gradient in rate of metabolism may be mentioned, although it is not clearly shown in the tables. In general, the degree of rejuvenescence in recon-

stitution is greater in posterior than in anterior pieces of the same size, provided of course that both produce new whole animals. The amount of reorganization in the reconstitution of a piece undoubtedly increases as the level of the piece becomes more posterior. The amount of new tissue behind the head and the degree of change in the alimentary tract both increase from anterior to posterior pieces. In other words, the farther from the head a given region of the body is, the greater the alteration it must undergo when brought into direct relation with a dominant head region as every piece is when a head forms at its anterior end.

#### IV. SUMMARY

1. A temporary increase in rate of metabolism, a 'stimulation' lasting at least several hours, results from section.

2. The temporary increase in rate of metabolism following section varies in amount inversely as the length of the piece, long pieces being very slightly or not at all stimulated and short pieces strongly stimulated.

3. The temporary increase in rate of metabolism following section also varies in amount according to the region of the body which the piece represents, being least in most anterior and increasing in successively more posterior pieces. In the shorter pieces this difference is sufficient to increase the rate of posterior above that of anterior pieces.

4. The differences along the axis in the stimulation from section are merely special expressions of the axial gradient and the antero-posterior dominance which results from it.

5. An inverse relation exists between the frequency of head formation and degree of stimulation of pieces following section. The shorter or the more posterior a piece the greater the degree of stimulation and the less the frequency of head formation. The nature of this relation will be considered after presentation of further facts.

6. The temporary increase in rate of metabolism in the piece after section is followed by a gradual fall. Twelve hours after section the rate of metabolism in the pieces may be as low as, or

lower than, that in corresponding regions of intact animals. During the following thirty-six to forty-eight hours the rate of metabolism remains about the same in the pieces.

7. After three or four days the rate of metabolism in the pieces begins to rise slowly as reconstitution proceeds. This rise in rate is relatively permanent and constitutes physiological rejuvenescence. It disappears only as the animal increases in size and becomes older with feeding. Its amount depends upon the degree of reconstitucional change and the size and region of the piece. Provided complete reconstitution occurs the degree of rejuvenescence is greater in posterior than in anterior and in small than in large pieces.

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## ON THE STRENGTH AND THE VOLUME OF THE WATER CURRENTS PRODUCED BY SPONGES<sup>1</sup>

G. H. PARKER

Ever since Grant discovered the currents produced in water by living sponges, this activity has been a matter of interest to the naturalist. The arrangement of the canals within the body of the sponge, the disposition of the motor elements or choanocytes, and the control of currents by means of sphincters have all received much consideration. Very little attention, however, has been given to the currents themselves, their strength and volume.

In a paper published in 1910 in the eighth volume of *The Journal of Experimental Zoölogy*, I recorded the fact that in *Stylotella*, a sponge from the coast of North Carolina, the pressure of its currents was equal to that of a column of water from 3.5 to 4 mm. in height. This sponge is of small size and generates only a slight disturbance in the surrounding water as compared with what may be seen in the vicinity of large sponges in the tropical and subtropical seas. Here the water often wells up so abundantly from the sponge as to deform the surface of the sea much as a vigorous spring deforms that of a pool into which it issues. Sponges of this description are frequently met with in the waters about the Bermuda Islands, and, during a recent sojourn at the Bermuda Biological Station, I took the opportunity of measuring the currents from some of these organisms.

The method that I used in making these measurements was the same as that which I had previously employed for *Stylotella*. A whole sponge or a large portion of one was transported in a tub or bucket to the laboratory and a glass tube of appropriate size was tied securely into one of its oscula. Care was taken to select for this purpose an osculum whose canal system had been unin-

<sup>1</sup> Contributions from the Bermuda Biological Station for Research, No. 32.



jured in detaching the sponge from its natural base or, if it had been necessary to break open the canal system anywhere, such breaks were closed by ligatures of soft string. The tube with the sponge attached was then held rigidly in place in the bucket of water and, after five minutes or so, the height of the water in the tube over that in the bucket was measured by means of a millimeter rule attached to the side of the tube. Without disturbing the tube and its attached measure, the canal system of the sponge was then cut open so that the water in this system was in free communication with that in the bucket. The water in the tube immediately fell and, after it had come to a constant level,

TABLE 1

*Showing pressures in millimeters of seawater exhibited by the currents of seven species of sponges from the Bermuda Islands*

NAME OF SPONGE	HEIGHT OF COLUMN OF SEAWATER IN MILLIMETERS FOR FIVE DETERMINATIONS					AVERAGE HEIGHT
Tethya sp. Close to <i>T. seychellensis</i> (E. P. Wright).....	2.5	2.0	2.5	2.0	2.0	2.2
Spirastrella sp. Close to <i>S. vagabunda</i> , var. <i>tubulodigitata</i> Dendy.....	2.5	2.0	2.0	2.5	2.0	2.2
Pachychalina sp.....	1.0	2.0	1.5	1.0	1.0	1.3
Spinosella sororia (Duch. et Mich)	3.0	2.5	3.0	3.0	3.0	2.9
Tedania sp.....	2.0	2.0	2.5	2.0	2.0	2.1
Stelospongia sp.....	2.5	2.0	2.0	2.0	2.0	2.1
Hircinia variabilis F. E. Schulze..	3.0	3.0	2.0	2.5	3.0	2.7

a reading of its height was again taken. The difference between this reading and that made before the sponge was cut open was assumed to be a measure of the pressure of the water current produced by the sponge.

Determinations of the current pressures by the method just described were made in seven species of sponges, and the details of of these measurements are given in table 1. At least five determinations were made for each species. I am under obligations to my friend, Prof. H. V. Wilson for the identification of the sponges upon which these determinations were made.

The surprising feature of the determinations in the table is that they all indicate a very low current pressure, lower even than that

of *Stylotella*. Notwithstanding this general lowness of pressure one of these species, *Spinosella sororia*, the common Bermuda finger-sponge, produces in the shallow sea a conspicuous deformation of the natural surface of the water, and this deformation can also be observed in specimens that have been transported to the laboratory. As *Spinosella* was a very satisfactory species to work with, a determination of the volume of water that passed through a single finger of this sponge was attempted. This was made by sinking the tube that had been fastened into the osculum of a finger of *Spinosella* under the surface of the seawater and thus allowing the water driven by the sponge to escape. The escaping current deformed the surface of the water in the bucket in much the same way as is often seen over a vigorous sponge finger in its natural position in the sea. The rate of the flow of water from the finger was then determined by measuring the velocity of floating particles, such as carmine and so forth, that were carried up the tube by the water current. This proved to be very close to 20 mm. in five seconds. As the diameter of the tube was 17 mm. the finger must have discharged a little over 4.5 cc. of water in five seconds. At this rate the discharge would amount to some 78 liters a day. This finger measured about 10 cm. in length and averaged 4 cm. in diameter; its osculum had a diameter of about 2 cm. *Spinosella* colonies often consist of as many as twenty such fingers and, assuming that all the fingers work at the same rate as the one upon which the measurements were made, such a colony as a whole would strain in a day about 1575 liters of seawater or over 415 gallons.

Since all the measurements recorded in the table were made upon sponges that had been removed from their natural attachments and transported to the laboratory and since the effect of such treatment is known to retard rather than accelerate the currents, the results therein recorded are believed to be below rather than above the actual working capacity of the several sponges. This opinion is borne out by an observation made on *Spirastrella* for which I am indebted to Mr. W. J. Crozier and Mr. D. H. Wenrich. A specimen of this sponge was found partly exposed at low water. Although the osculum was out of water, there was

still a free flow from it. A cut was made on the side of the sponge at the level of the outer seawater and the sponge was taken to the laboratory. Close measurements showed that the edge of the osculum over which the water lifted by the sponge had been flowing was 4 mm. above that of the sea level, demonstrating that this sponge in the undisturbed condition in which it was found had been overcoming a pressure of 4 mm. of seawater. As the highest pressure that had been obtained from *Spirastrella* in the laboratory was 2.5 mm., it seems probable that the laboratory determinations for all the species tested are a little below the actual maxima for this form of activity, though, for reasons already given in my former paper on *Stylotella*, they cannot be much below this maximum.

From these observations it is clear that the currents produced by sponges consist of relatively large volumes of water flowing at low pressure and that currents of this kind are capable of producing such deformations of the surface of the sea as can be observed above large sponges. It would be interesting to ascertain what proportion of the suspended material in the seawater is screened out as the current passes through the sponge, but no observations on this point were made.

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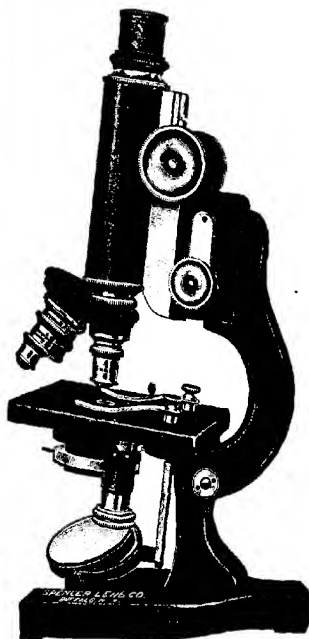
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## CONTENTS VOLUME 16

### NO. 1 JANUARY

E. J. LUND. The relations of Bursaria to food. Eight figures.....	1
ALICE M. BORING AND RAYMOND PEARL. The odd chromosome in the spermatogenesis of the domestic chicken. Six plates.....	53
CHARLES PACKARD. The effect of radium radiations on the fertilization of <i>Nereis</i> .....	85
JOHN C. PHILLIPS. A further study of size inheritance in ducks with observations on the sex ratio of hybrid birds. Seven charts.....	131
GEORGE L. STREETER. Experimental evidence concerning the determination of posture of the membranous labyrinth in amphibian embryos. Thirty-six figures.....	149

### NO. 2 FEBRUARY

E. C. MACDOWELL. Multiple factors in Mendelian inheritance.....	177
HENRY LAURENS. The reactions of normal and eyeless amphibian larvae to light. Two figures.....	195
GEORGE ALFRED BAITSELL. Experiments on the reproduction of the hypotrichous Infusoria. II. A study of the so-called life cycle in <i>Oxytricha fallax</i> and <i>Pleurotricha lanceolata</i> . Sixteen figures (one plate).....	211
LORANDE LOSS WOODRUFF. On so-called conjugating and non-conjugating races of <i>Paramecium</i> . One figure.....	237
ARTHUR WILLIAM MEYER. The supposed experimental production of hemolymph nodes and accessory spleens. V. Studies on hemal nodes.....	241

---

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### 1914 VOLUME 24 1914

#### NO. 1 FEBRUARY

C. JUDSON HERRICK. The cerebellum of <i>Necturus</i> and other urodele amphibia. Thirty figures.....	1
M. R. CHASE AND S. W. RANSON. The structure of the roots, trunk and branches of the vagus nerve. Twenty figures.....	31
ELBERT CLARK. Regeneration of medullated nerves in the absence of embryonic nerve fibers, following experimental non-traumatic degeneration. Thirty-two figures....	61



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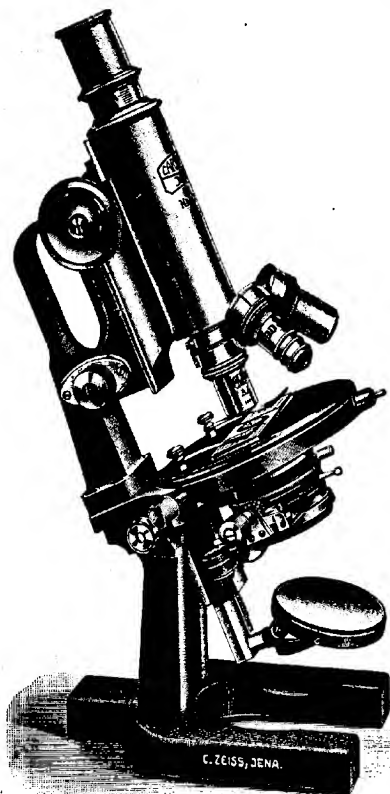
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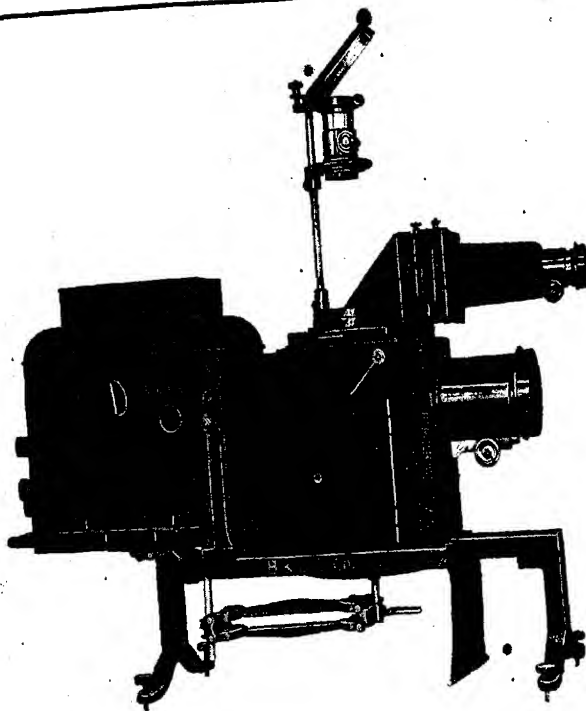
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# MODES OF INHERITANCE IN TELEOST HYBRIDS

H. H. NEWMAN

*From the Hull Zoological Laboratory, University of Chicago, and the Marine  
Biological Laboratory, Woods Hole*

THIRTY-EIGHT FIGURES

## CONTENTS

Introduction.....	447
Historical review of the work of Appellöf, Moenkhaus, Kammerer, Bancroft and Newman on teleost hybrids.....	448
New experiments: Reciprocal crosses between species of the genus <i>Fundulus</i> and the genus <i>Cyprinodon</i> .....	457
1. Material and methods.....	457
2. Tabulations of data.....	459
3. Summary and discussion of data.....	467
Inheritance of pigment characters in <i>Fundulus</i> hybrids.....	475
1. A statement concerning pigment characters in <i>Fundulus</i> species.....	475
2. Tabulations of data.....	478
3. Summary and discussion of data on pigment inheritance.....	482
General summary of conclusions.....	486
1. The nature of the influence of foreign spermatozoön on cleavage and early development.....	486
2. Harmful versus beneficial effects of foreign spermatozoön.....	487
3. Phylogenetic relationship of parent species and success in development of hybrids.....	487
4. Modes of inheritance in teleost hybrids and the Mendelian hypotheses of dominance and segregation.....	488
5. Inheritance in reciprocal crosses.....	489
Bibliography.....	489

## INTRODUCTION

The present contribution has a twofold purpose, to review and discuss the literature on teleost hybrids and to present a new body of experimental data, which seem to throw added light on various mooted questions and to lead to rather definite conclusions concerning certain problems of inheritance.

As material especially available for the study of the early stages of hybrid development the teleosts are unexcelled by any

other group of animals known to the writer, although a much larger number of investigators have chosen to work with echinoderms. Teleosts possess decided advantages over the echinoderms in that they intercross in practically any combination and without the aid of the reagents used in artificial parthenogenesis, which are necessary in order to cross echinoderms, and which may affect the character of development and thus interfere with the study of heredity. It is, moreover, a simple matter to rear the young of teleosts to a stage in which many definitive characters can be studied, while hybrid echinoderms have never to my knowledge been reared beyond a comparatively early larval stage. In addition to these two fundamental advantages teleosts afford especially good material for this type of investigation because the large number of species in any neighborhood give a very wide range of possible combinations; because the eggs and young are large and easily handled; and because the contrasting characters are more numerous and more clearly defined than are those of echinoderms.

HISTORICAL REVIEW OF THE WORK OF APPELLÖF, MOENKHAUS,  
KAMMERER, BANCROFT AND NEWMAN ON TELEOST HYBRIDS

The earliest published work on teleost hybrids that has come to my attention is an important paper by Appellöf that appeared early in 1894. I regret that this paper escaped my notice until after the publication of my two former papers on *Fundulus* hybrids, for it would have been highly suggestive for certain phases of my own work. The paper, although rather generally overlooked by investigators, should be of considerable interest to all those who are engaged in the study of the developmental mechanics of heterogenic hybrids. Appellöf was probably the first to attempt crosses between very distantly related species. His combinations include such ill-assorted matings as the stickleback and the flounder, the cod and the cunner, the cod and the flounder and others equally bizarre. In each case he found that, even though a mature hybrid between the two parent types be unthinkable, development in the cross fertilized eggs proceeds

for a considerable period, in most cases through the cleavage period, in a normal manner. In all cross bred eggs the cleavage seemed to be identical with that of pure bred eggs except that in rare cases the time rate of the cleavage process was markedly retarded. This was especially noticeable in the hybrids from *Spinachia* eggs fertilized with *Gasterosteus* sperm.

An observation of Appellöf's, which to my mind is of considerable morphological significance, is to the effect that the most critical period in the development of heterogenic hybrids is the period of transition from cleavage to germ layer formation—the period of 'gastrulation.' In comment upon this finding I may say that my own results have for a number of years forced me to similar conclusions and that I have on hand an investigation dealing at some length with this point. It is of more than passing interest to note how this idea lines up with the rather prevalent opinion that the process of cleavage is purely a function of the egg, and that the spermatozoön, though coöperating with the egg nucleus in the mitoses of cleavage, does not begin to influence the process of heredity until during the formation of the gastrula. It would appear that even a very distantly related sperm may stimulate development in an egg and coöperate harmoniously with the latter through the period of cell multiplication, but that when the hereditary functions of the germinal elements come into play discordant chemical interactions take place that effectually disrupt developmental harmony. This barrier to further development I have found to be less sharply drawn than Appellöf supposed, as will become evident when the data subsequently presented in this paper claim the reader's attention.

●Next in chronological order comes the work of Moenkhaus ('94) in which he relates the results of his crosses between *Fundulus heteroclitus* and *Menidia notata*. This work had as its object the study of the behavior of maternal and paternal chromatin in hybrid eggs and embryos in which the chromosomes of the parental species were visibly different in form. The observations show that the paternal chromosomes retain their identity in the zygote and for a considerable period remain in a close



group sharply marked off from the maternal group. Subsequently, however, the two types intermingle, though still retaining their specific characters. The rate of cleavage was, according to Moenkhaus, the same as in pure bred eggs of the same species. It is a noteworthy fact that when *Fundulus* eggs are fertilized with *Menidia* sperm, the period of gastrulation is safely passed and a well-defined, though short and defective embryo is formed although the two species represent two distinct orders of teleosts.

Kammerer ('07) published a rather extensive account of hybridizing two species of perch, *Perca fluviatilis* and *Acerina cernua*. In brief his report is as follows. In the spring of 1905 a fisherman brought to the laboratory two specimens of an unknown type of perch, which, at the suggestion of Doctor Przibram, were adjudged to be hybrids between *Perca fluviatilis* and *Acerina cernua*, because in a number of respects they appeared to be intermediate between these two species. In order to test the correctness of this diagnosis it was decided to intercross the hypothetical parent species in order to determine whether the hybrids resembled the questionable wild individuals. It was found that the two species did cross successfully and that a number of young fish hatched and grew well for about six months, at which time they showed some characters which slightly resembled those of the supposed adult wild hybrids. The latter were successfully crossed, since they were both fertile females, with males of both *Perca* and *Acerina*, the young resembling closely the paternal species in each case. This is the only case on record, so far as I am aware, of fertile hybrids being produced by crossing two genera. It is rare, in fact, to find undiminished fertility in hybrids between two closely related species of the same genus, and even inter-varietal hybrids frequently show incomplete fertility. Had Kammerer reared to maturity and successfully bred the unquestioned hybrids which he obtained he would have made good his case, but as the matter stands there is considerable cause to doubt the validity of his conclusions.

The chief value of the paper in the present discussion lies in the fact that there is presented a considerable array of data,

tending to show that in practically all characters examined the hybrids reared in the laboratory are strictly *intermediate* between those of the parent species. This is true for such matters as numbers of scales in a row, numbers of fin-rays, numbers of teeth, and other integral variates. Characters involving dimensions show a similar *blending* between the two species. No mention is made of the discovery of any type of inheritance suggestive of Mendelian dominance.

In addition to the crosses mentioned above Hammerer made a number of heterogenic crosses between teleost species, the results of which led him to conclude that the degree of success in the development of hybrids depends not upon the closeness of relationship of the parent species but upon their similarity of habitat. The fallacy of this conclusion is evident in view of the success of Moenkhaus and the writer in cross fertilizing practically any two species of teleosts.

The first attempt to follow in detail the development of teleost hybrids was made by the writer (Newman '08). This paper dealt exclusively with the reciprocal hybrids between *Fundulus heteroclitus* and *Fundulus majalis*. The former species develops nearly twice as rapidly as the latter and the reciprocal hybrids both have a developmental time rate intermediate between those of the parent species, though more nearly like that of the egg than that of the sperm species. All characters involving the time factor show blended inheritance, but many characters not dependent on the time factor were shown to exhibit more or less typical Mendelian dominance. Some of the dominant characters noted were as follows: *F. heteroclitus* type of chromatophore dominant over that of *F. majalis*; size of young on hatching or at the time of maximum development that of *F. heteroclitus* in both reciprocal crosses; the rate of cleavage nearly pure maternal; the viability of young hybrid larvae, when capable of hatching, equal to or greater than that of the more viable species, *F. heteroclitus*; resistance to lack of oxygen or presence of carbon dioxid of *F. heteroclitus* egg hybrid as great or greater than that of the more resistant species; susceptibility to these reagents greater in *F. majalis* egg hybrids than in the more susceptible

species, *F. majalis*; the depth of pigmentation of young *F. heterooclitus* egg hybrids as pronounced or more so than the darker species, *F. heterooclitus*. These cases in which the hybrids seemed to show an exaggeration of dominance exemplify the phenomenon of 'hyperdominance.' In view of the fact that at the time when this paper was written the new Mendelism was sweeping all before it, I took pains to emphasize especially all types of inheritance which appeared to me to be essentially non-Mendelian. This statement was taken advisedly in the hope of checking the growing impression that all inheritance was on analysis Mendelian, and that cases of apparent blending, even in the  $F_1$  generation of hybrids, was the result of incomplete analysis of the factors involved.

Chief stress was laid upon the importance of studying heredity as a *process*, of observing the genesis of characters rather than limiting observation to the definitive conditions as they appear in adults. In this connection it was noted that maternal and paternal influences showed alternating periods of predominance, so that a character might appear as a dominant at one time only to be superseded by the character that had apparently been recessive. The definitive condition represents only the end result of a struggle between opposed parental tendencies whose ups and downs may be observed from day to day in developing hybrid embryos.

The question as to whether the foreign sperm exercises any influence upon the rate and character of early cleavage was in this paper answered in the negative for the reason that in all early experiments the differences were very small and not always in the same direction.

This paper was followed nearly two years later by another dealing with the same species of fish (Newman '10), in which the question as to the influence of the spermatozoön on early development was reexamined. Very searching statistical methods were applied to the relative rates of early cleavage in pure and hybrid *F. majalis* eggs. It was found as the result of five experiments involving very large numbers of eggs, that there was a slight but constant acceleration of cleavage as the result of the

use of foreign sperm. On this score I took occasion to criticise the position of Conklin, Godlewski and others, who, on the basis of the work done on echinoderm hybrids, had declared that the early development of hybrids is purely maternal and that it is only in stages later than the gastrula that the influence of the spermatozoön begins to make itself felt. There was in my experiments a real effect of foreign sperm on the rate of early development and this effect was not due to a more speedy impregnation of the egg membrane by the foreign sperm head, as was shown by the experiment in which all spermatozoa in both hybrid and control cultures were destroyed with distilled water within less than five minutes after insemination. By this time all spermatozoa had entered the eggs, those of the foreign and native species having entered with equal promptness.

These results were criticised by Godlewski ('11) because they were not confirmed by cytological studies. He did not explain, however, just what he meant by a cytological confirmation of a statistical treatment involving many thousands of eggs, no small sample of which could be expected to show anything of value. On the basis of what seemed to be a demonstration that foreign sperm actually effects a slight change in the rate of early cleavage, the writer entered upon a general discussion of the rôle of the spermatozoön in early development and came to the conclusion that, in addition to initiating development, the paternal germ plasma reacts almost immediately with that of the egg so as to change the rate and character of metabolism and thus accelerate or retard cleavage. Naturally the cytoplasmic organization of the egg imposes upon the young embryo certain morphological restrictions, but in all those matters that have to do with rate of chemical reaction the spermatozoön begins to exert an influence as soon as fertilization occurs, as soon as the two ontogenies are engrafted one upon the other. The work of Godlewski, Baltzer and others, who had experimented with echinoderm hybrids, was cited by the writer in confirmation of his conclusions and it is to be regretted that, in citing Godlewski's results certain misstatements were made, for it was in response to these inadvertent inaccuracies that Godlewski took the whole

paper rather severely to task. The fundamental contention was, however, scarcely touched upon by this critic, as was shown by the writer (Newman '11) in a reply to Godlewski, in which the minor inaccuracies in reporting the latter's results were admitted and differences of interpretation were brought clearly upon a controversial plane.

It may then be considered as fairly well established that, *at least in the case of hybrids between closely related species, the spermatozoön exercises at first a slight and then a progressively more pronounced influence upon the rate of early development, but fails to exercise any really hereditary function until the embryo begins to differentiate tissues and organs.* This point of view will, I believe, be justified by the new data herewith presented. This somewhat radical change of front on my part is the result of several years of study and has been forced upon me especially by the experiments about to be reported, the results of which are herewith to some extent anticipated.

In the Proceedings of the Indiana Academy of Science for 1910, published a year later, Moenkhaus reports upon an extensive series of experiments dealing with "Cross fertilization among fishes." These range from extreme heterogenic crosses between different orders of teleosts to those between closely related species of the same genus. A useful summary of the chief facts and conclusions is furnished and is here quoted:

1. The eggs of any of the species of teleosts tried may be impregnated by the sperm of any other species tried.
2. The number of eggs fertilized is usually great, i.e., 75 per cent or more. This bears no relation to the nearness of relationship of the two species concerned.
3. Normal impregnation is the rule, di- and polyspermy being the exception.
4. Development in its early stages proceeds normally, the deleterious effects of the two strange sex products upon each other showing only at later cleavage or subsequently.
5. The rate of development in the early cleavage stages is always that of the egg species. Any effect of the strange sperm upon the rate of development shows itself by slowing the process regardless of whether the rate of the sperm species is faster or slower than the egg species. A period of great mortality in the developing hybrids is gastrulation.

6. If the heart is formed, although it pumps no blood, the embryo may remain alive for a considerable period, yolk absorption taking place to a varying degree. If the heart handles blood and the blood vessels are differentiated, the embryo is likely to develop to the point of hatching.

7. The numerous abnormalities appearing in the hybrid embryos are due to a deterioration in the developmental processes, resulting probably from the poisonous action of the sex products upon each other.

8. The success of the hybrids, i.e., the stage to which a given hybrid will develop, is correlated with the nearness of relationship of the two species used.

9. The mixing of unrelated sex products is looked upon as analogous to the transfusion of unrelated bloods, the more distantly related the two species concerned the greater their toxicity.

For the most part the results of Moenkhaus confirm the earlier results of Appellöf and extend the horizon of our knowledge of the field in question. I am unable, however, to accept any of the last six conclusions listed above, and will present my reason for thus dissenting after presenting my new results.

Bancroft ('12) has made the most recent contribution to the literature on the hybridization of teleosts. He repeated the experiments published by the present writer in 1908, intercrossing *Fundulus heteroclitus* and *F. majalis*, paying especial attention to the heredity of pigmentation. The avowed object of the experiments was to reexamine the hybrids "from the Mendelian and physiological points of view." Bancroft confirms my observations and in most points agrees with my conclusions. In his search for evidences of Mendelian dominance he overlooks many *intermediate* conditions. In one instance he attempts to explain what had seemed to me a perfect case of blended inheritance as the result of the interaction of two separate unit factors. He confirms my results and conclusions very exactly when he concludes that "in general, characters connected with rate of development show blended heredity and it may be that such characters are so intimately associated with extra-nuclear substances such as the yolk that complete dominance is not obtained."

It is noteworthy, in view of Moenkhaus's statement "that any effect of the strange sperm upon the rate of development shows itself by slowing the process of development regardless of whether

the rate of the sperm species is faster or slower than the egg species," that Bancroft takes the position that:

As regards the rate of development of the embryos my observations confirm those of Newman on most points. The development of the *F. heteroclitus* egg hybrid was shown to be slower than that of its maternal parent; and the development of the *F. majalis* egg hybrid, during the early stages, was faster than that of the pure *F. majalis*. After hatching the *F. heteroclitus* egg hybrid seemed more vigorous and grew faster under like conditions than either of the pure forms.

It is somewhat strange that Moenkhaus failed to note these facts for he must have performed the experiment a number of times. Bancroft's paper adds materially to our knowledge of the heredity of pigment in teleost hybrids. His analyses of these processes is more nearly adequate than that of any other writer. In the general discussion of new data I shall have occasion to come back to this paper for more detailed treatment.

This then is the history of the development of our knowledge of heredity in teleost hybrids. From this array of facts and theories it is possible to extract certain well-defined problems and unsettled questions, which it is my intention to put to the test of further experiment. A list of some of the more important questions is herewith introduced in order to clarify the issue and to focus the attention of the reader upon the problems involved.

1. At what point in the development of an embryo does the sperm begin to exercise an hereditary as opposed to a merely chemical effect upon the character of development?

2. Is the rate of cleavage 'inherited,' in a strict sense, or is it merely a function of egg size and of yolk content?

3. Is the effect of foreign sperm to be looked upon as necessarily deleterious, and hence toxic, or is the effect bad only when there arises so pronounced a disharmony of the two engrafted ontogenies that no blending or compromise can be reached?

4. Is it true that all characters concerned with or resulting from the rate of development are inherited in the blended fashion? Is rate of development strictly an hereditary character?

5. Is the degree of success in the development of hybrids in any way closely correlated with the nearness of relationship of

the two species used? If not, upon what factors does success in crossbreeding depend?

6. Are structural or physiological characters inherited in the exclusive or in the blended fashion in the first generation of hybrids?

7. What is the function of the spermatozoön in early development? Is its initial effect merely equivalent to that exercised by mechanical and chemical agents used in artificial parthenogenesis, or is this only part of its function?

8. Is there any truth in the analogy suggested by Moenkhaus between the effects resulting from mixing strange sex products and those resulting from the transfusion of unrelated bloods?

Each of these questions will receive its proper share of attention elsewhere in the paper.

#### NEW EXPERIMENTS: RECIPROCAL CROSSES BETWEEN SPECIES OF THE GENUS *FUNDULUS* AND THE GENUS *CYPRINODON*

##### 1. MATERIALS AND METHODS

The four species of fish used in the present experiments belong to two genera of the family Poeciliidae and are common in the waters about Woods Hole. They are *Fundulus heteroclitus* (figs. 1 and 2), *F. majalis* (figs. 3 and 4), *F. diaphanus* (figs. 5 and 6), and *Cyprinodon variegatus* (figs. 7 and 8). *F. heteroclitus*, *F. majalis* and *Cyprinodon* are marine or brackish water species, while *F. diaphanus* is strictly a fresh water form though tolerant of slightly brackish water. In some regions *F. heteroclitus* is found with *F. diaphanus* in fresh water ponds into which the sea flows at high tide. The eggs of *F. majalis* are the largest, averaging about 2.7 mm. in diameter, those of *F. diaphanus* come next with an average diameter of 2.3 mm., those of *F. heteroclitus* average 2 mm., and those of *Cyprinodon* are much the smallest, averaging scarcely 1.5 mm. The yolk of *F. majalis* eggs is denser and of deeper yellow color than that of the other species; that of *F. heteroclitus* is denser and yellower than in *F. diaphanus* but less so than in *F. majalis*; that of *Cyprinodon* is almost colorless.



During the months of June and July all of these species are spawning in the Woods Hole region and little difficulty is experienced in obtaining abundant material for crossbreeding. The most successful experiments were those in which insemination was accomplished by the dry method. In this way it was possible to avoid exposing either of the sexual products to waters of harmful concentration, for eggs were stripped from well dried females into bowls containing no water and they were mixed with fresh milk or macerated testis. After allowing a few minutes for the sperm to impregnate the eggs, water of the proper kind was added and excess sperm washed out. It was found best to rear the eggs of *F. heteroclitus*, *F. majalis* and *Cypriodon* in sea-water and those of *F. diaphanus* in fresh water. All thrive well in about 25 per cent sea-water, but do no better than when the two kinds of natural water are used. Likewise all four kinds of eggs thrive in fresh water, but the eggs of *F. diaphanus* do not do well in natural sea-water, in which they undergo various degrees of plasmolysis.

No less than three crosses of each type were performed and careful comparisons were made possible through the frequent camera drawings of individuals and details. The illustrations herewith published are taken from camera drawings which seem to represent the most typical condition. The method of presenting the data is as follows: A chronology is given for each cross and beside it in an adjacent column are given the details of synchronous stages in the development of the control or pure bred eggs of the maternal species. Only such details are mentioned as seem of particular significance as diagnostic of relative rates of development or types of inheritance involved. In order that repetition may be avoided the chronologies for the reciprocal hybrids between *F. heteroclitus* and *F. majalis* are omitted, and the reader is referred for the facts to the previous paper in which these crosses were first dealt with (Newman '08). The references to details of pigmentation are intended to be merely suggestive, since the study of pigment inheritance forms a special topic for subsequent discussion.

## 2. TABULATION OF DATA

### a. Crosses between species of the same genus .

TABLE I

Comparing the hybrids from *F. diaphanus* ♀ × *F. heteroclitus* ♂ with pure bred *F. diaphanus* .

TIME	PURE <i>F. DIAPHANUS</i> (CONTROL)	<i>F. DIAPHANUS</i> ♀ × <i>F. HETEROCLITUS</i> ♂
2 hours	First cleavage	Same
6 hours	Advanced cleavage	A larger proportion in more advanced stages than in control
20 hours	Blastoderm with germ ring faintly defined, embryonic shield barely visible (fig. 10)	Germ ring well defined and well established embryonic shield (fig. 11)
2 days	Germ ring halfway around the yolk, embryonic axis well defined, no neural tube (fig. 13)	Germ ring nearly around yolk, embryo long and with brain and optic vesicles well defined (fig. 14)
3 days	Blastopore closed, embryo with short tail, lenses of eyes lenticular in form, mid-brain broadly open, no heart-beat or circulation, first chromatophores under hind-brain and on adjacent yolk	Blastopore closed, tail long, lenses spherical and partially enclosed in optic cup, entire brain closed, heart beating in majority of embryos, much more numerous and better defined chromatophores on brain and yolk
4 days	Heart-beat beginning to be established in nearly all embryos, no circulation, scattering stellate dark chromatophores on top and sides of brain, black yolk chromatophores scattered over whole yolk, a few small red yolk chromatophores of dull orange color (fig. 16)	Heart beating strongly, yolk and body circulation well established, pigmentation much more advanced in every particular than in control, red yolk chromatophores redder than in control (fig. 17)
5 days	Embryos about like those of pure <i>F. heteroclitus</i> at 4 days, heart-beat strong, circulation well established, head chromatophores all brownish and small, large black yolk chromatophores arranged along the principal vitelline vessels, red yolk chromatophores large, stellate, and dull red-dish brown	Embryos less markedly in advance of control than at 4 days, a few large black head chromatophores of the <i>F. heteroclitus</i> type have appeared along with numerous small brownish head chromatophores of the <i>F. diaphanus</i> type, red yolk chromatophores intermediate in color between the red-brown of the maternal and the brick red of the paternal species

TABLE 1 Continued

TIME	PURE <i>F. DIAPHANUS</i> (CONTROL)	<i>F. DIAPHANUS</i> ♀ × <i>F. HETEROCALITUS</i> ♂
1 week	Embryo as in fig. 19, almost even with the hybrid strain in stage of development, other details about as before	Embryos as in fig. 20, tail shorter than in control, other details as before
9 days	Embryos comparatively light colored, tail long and slender	Embryos much darker than control, decidedly larger and more advanced
12 days	None hatched	Nearly all hatched
13 days	A few hatched	All hatched and very active
14 days	About half hatched	
15 days	All hatched	
3 weeks	Larvae vigorous and would doubtless live indefinitely	Larvae larger and more active than control, evidently growing faster under like conditions

TABLE 2

Comparing the hybrids from *F. heteroclitus* ♀ × *F. diaphanus* ♂ with pure bred *F. heteroclitus*

TIME	PURE <i>F. HETEROCLITUS</i> (CONTROL)	<i>F. HETEROCLITUS</i> ♀ × <i>F. DIAPHANUS</i> ♂
2 hours	First cleavage	Same
4 hours	32 to 64-cell stages	Like control except that a small percentage of eggs are less advanced than any of the pure bred eggs, having 16 cells or less
20 hours	Advanced cleavage but no germ ring visible	Some embryos show the early steps in germ ring formation, others are less advanced than any of the control
52 hours	Blastopore closed or nearly so in all embryos, pale chromatophores appearing under hind-brain, a few red yolk chromatophores on yolk near head, no black yolk chromatophores	The normally developing embryos noticeably more advanced and more deeply pigmented than controls, <i>F. diaphanus</i> type of head chromatophores appearing on sides of mid-brain, a few black yolk chromatophores
3 days	Heart beginning to beat feebly in nearly all embryos, no vitelline circulation. A few black yolk chromatophores, and fewer head chromatophores	Heart beating quite strongly in a few embryos, the majority about as in control, a considerable percentage of abnormal, retarded embryos derived from the eggs that showed lagging cleavage at 4 hours
4 days	Vitelline and body circulation established and all types of chromatophores abundant	Almost identical with control in stage of development except the abnormal specimens, which from here on will be ignored as they never hatch; the normal embryos more heavily pigmented than control
5 days	Embryos nearly as advanced as those of <i>F. diaphanus</i> at 6 days	Very much like control; pigment characters given in detail later
1 week	Body shorter and stouter than in hybrid, otherwise little different except in details of pigmentation	Body rather more slender than control, a few more deeply pigmented than any of the controls
13 days	A few hatched	The majority hatched
14 days	All hatched	All hatched except abnormal specimens
3 weeks	Larvae vigorous	Larvae indistinguishable from control except in details of pigmentation

TABLE 3

*Showing development of hybrids from F. diaphanus ♀ × F. majalis ♂; for control see first column of table 1*

2 hours	First cleavage like control
6 hours	A smaller proportion of the more advanced stages of cleavage than in control
20 hours	Blastoderm much smaller than in control, germ ring scarcely distinguishable, no embryonic shield (fig. 9)
2 days	Germ ring one-third around the yolk, embryonic shield small and only slightly elevated from germ ring, embryonic axis not established (fig. 12)
3 days	Blastopore still open, in some cases germ ring is only three-fourths around yolk, optic vesicles flat with no optic cup, no lenses, no enlargement of hind-brain, no chromatophores
4 days	Embryo much less advanced than in control, no heart-beat, no body or yolk pigment (fig. 15)
5 days	Embryo about as advanced as control was at 4 days, chromatophores on head and yolk few as compared with control and all more delicately branched as in paternal species, no vitelline circulation but the majority of embryos have heart-beat established. Nearly half of the embryos appear at this time as if they were abnormal
1 week	The normal embryos have developed very rapidly since the establishment of vitelline circulation and are now nearly on a par with the control; many specimens, however, have failed to establish vitelline circulation and appear anemic and deformed
9 days	Even the more advanced embryos have lagged behind control; a number of abnormal embryos have died from anemia, the most pronounced differences between hybrids and controls have to do with pigmentation
12 days	None hatched
13 days	None hatched
14 days	A very few hatched
15 days	About a fourth hatched, larvae not very active
16 days	Nearly all of the normally developing embryos hatched; a few have died without hatching
3 weeks	Nearly all larvae dead, the few that have survived are not as large or as vigorous as the pure bred larvae of the same age, living under identical conditions

TABLE 4

Comparing hybrids from *F. majalis* ♀ × *F. diaphanus* ♂ with pure *F. majalis*

TIME	PURE <i>F. MAJALIS</i> (CONTROL)	<i>F. MAJALIS</i> ♀ × <i>F. DIAPHANUS</i> ♂
3 hours	In 2- and 4-cell stages	Same
6 hours	16 to 32 cells	Mostly 32-cell stages
22 hours	Blastoderm beginning to form faint germ ring	Blastoderm much broader than in control, germ ring well defined, embryonic shield beginning to appear as a thickening of germ ring
2 days	Germ ring about halfway around yolk, triangular embryonic shield, embryonic axis a thin line	Germ ring in more advanced embryos as much as two-thirds around yolk, embryonic axis more sharply defined than in control
3 days	Germ ring nearly around yolk but blastopore still widely open, optic vesicles flat without optic cups, no lenses, no mesoblastic somites	Blastopore closed, optic cups formed, lens flat, 4 to 7 somites
4 days	Embryos with many somites, well differentiated brain vesicles, no heart-beat, no circulation, no pigment	Embryos smaller but more advanced than control, no heart-beat or circulation, scattering slender black chromatophores on yolk, none on body
5 days	Embryo large, heart-beat and circulation well established, no chromatophores	Embryos about as advanced as control in general development, but much smaller in size, all show the paternal influence in pigmentation, since there are both black and red chromatophores on yolk and black chromatophores of the <i>F. diaphanus</i> type, or nearly so, on the sides of the hind-brain
1 week	Embryos very much larger than hybrids, a few slender black chromatophores on sides of mid- and hind-brains, slender black chromatophores on yolk; no red yolk chromatophores	Hybrids much smaller, eyes paler, head chromatophores almost pure paternal in type, black yolk chromatophores almost pure paternal, red yolk chromatophores intermediate in form and color between two parental types. Red chromatophores in three rows on side of body as in <i>F. diaphanus</i>

TABLE 4—Continued

TIME	PURE <i>F. MAJALIS</i> (CONTROL)	<i>F. MAJALIS</i> ♀ × <i>F. DIAPHANUS</i> ♂
9 days	Head and body chromatophores all very finely branched and scattering, yolk chromatophores both red and black very slender	On the head the <i>F. diaphanus</i> type of chromatophore predominate, but there are always some of them with a tendency to branch like those of the maternal species; red yolk chromatophores much larger than in control and of an intermediate form and color. Body of embryos beginning to appear anemic on account of the interference with circulation produced by stretching of the heart, as in other <i>F. majalis</i> egg hybrids described elsewhere
18 days	Several hatched	Very little change since 9 days except that a number of the weaker embryos have died
20 days	All hatched	Many dead, others very feeble. There has been very little advance since the first week of development except in the case of the chromatophores. None hatched in any experiment

## b. Crosses between two genera

TABLE 5

*Hybrids between F. diaphanus ♀ × Cyprinodon variegatus ♂*

3 hours	About half of the eggs fertilized and in 2- and 4-cell stages, a few obviously polyspermic, others indistinguishable from control
5 hours	Advanced cleavage of regular character in the majority of developing eggs, but polyspermic eggs show great irregularity and considerable cytolysis
18 hours	All monospermic eggs normal in appearance but less advanced than control, the beginnings of cytolysis in a few blastodiscs
24 hours	All eggs show cytolysis of marginal cells of blastodisc, indicating that development has ceased and disintegration has set in. The limit of development in this cross seems to be the end of the cleavage period although a few embryos have begun to show the first indications of a germ ring, indicating that gastrulation or germ layer differentiation had begun but could progress no further

TABLE 6

*Hybrids between Cyprinodon variegatus ♀ × F. diaphanus ♂*

2 hours	About 75 per cent of eggs cleaving and 4- to 8-cell stages. Cleavage regular without any indications of polyspermy
4 hours	All fertilized eggs in advanced cleavage stages, like control but less advanced
20 hours	A few of the eggs that had cleaved are dead and partly disintegrated, but the majority have reached the end of the cleavage period and are beginning to show evidences of gastrulation. All are decidedly less advanced than control, in which all eggs are in germ ring stages
26 hours	Nearly half of the embryos that had reached the stage described above now show cytolysis, the others are still alive and in early germ ring stages
2 days	Only four embryos still alive. These have the germ ring one-third to one-half around the yolk, a triangular embryonic shield and a well-defined embryonic axis. Many embryos have died in early germ ring stages
3 days	Two embryos alive. These have blastopore nearly closed but show very little embryonic differentiation
4 days	Both embryos dead without having advanced beyond the condition noted on the previous day. It is possible for a few individuals of this cross to live practically through the period of gastrulation, but they are unable to enter upon embryonic differentiation. It is to be noted, however, that this cross succeeds much better than the reciprocal cross described in table 5

TABLE 7

*Hybrids between F. heteroclitus ♀ × Cyprinodon ♂*

2 hours	About 20 per cent of eggs cleaving. Polyspermy evident in nearly half of these eggs if one is to judge by the irregularity of blastomeres. The commonest irregularity consists of the production of two cells of very unequal size
5 hours	Nearly all that had cleaved are in advanced cleavage stages and appear to be quite normal. Evidently early irregularities have had little effect on the cell arrangements of this period
12 hours	Many eggs in advanced cleavage stages disintegrating. These are doubtless the polyspermic eggs. Others still normal in appearance
20 hours	All embryos dead, some evidently have but recently ceased to develop. These had succeeded in completing cleavage but show no signs of having entered upon gastrulation



TABLE 8

*Hybrids between Cyprinodon ♀ × F. heteroclitus ♂*

2 hours	A large proportion of eggs cleaving and in 4- to 8-cell stages; no signs of polyspermy
5 hours	A great variety of cleavage stages, from 8 to more than 32 cells, for the most part regular or nearly so
20 hours	A few embryos have reached the end of the cleavage period and have started to form germ rings. The majority have begun to disintegrate at the end of the cleavage period
2 days	All those that were developing at 20 hours now dead, several having reached a condition in which the germ ring was about half around the yolk and a flat embryonic shield with no visible embryonic axis had been differentiated. This cross is more successful than the reciprocal cross, but less so than when the same eggs are fertilized by the sperm of <i>F. diaphanus</i>

TABLE 9

*Hybrids between F. majalis ♀ × Cyprinodon ♂*

3 hours	About 25 per cent of eggs cleaving and in 2- and 4-cell stages, many irregular and some evidently polyspermic
6 hours	Only a very few eggs alive, others show various degrees of cytolysis
10 hours	All dead and disintegrating after reaching advanced cleavage stages Note: Another cross of these two species gave even less success than that here indicated

TABLE 10

*Hybrids between Cyprinodon ♀ × F. majalis ♂*

2 hours	Nearly all eggs cleaving in an apparently perfectly normal manner.
4 hours	A wide range of cleavage stages ranging from 8 to 32 cells. Those that are much retarded look unhealthy
20 hours	All but two embryos dead in advanced cleavage stages. The two that are alive are in early germ ring stages, showing flat embryonic shield. Both are much less advanced than control
2 days	The two embryos that were alive at 20 hours are now dead without having made much progress since the last observation. This cross is more successful than the reciprocal, but less successful than either of the other <i>Cyprinodon</i> egg hybrids

## 3. SUMMARY AND DISCUSSION OF DATA SHOWN IN TABLES

*a. Successful crosses*

Out of twelve crosses possible between the four species dealt with in this paper four have been successful in the sense that development up to hatching took place and more or less viable larvae were produced.

The most successful crosses are those between *F. diaphanus* and *F. heteroclitus*, in spite of the fact that the former is a fresh water species and the latter a marine species. These two species cross reciprocally with equal facility, producing in each case some larvae that hatch earlier than those from pure bred eggs of the maternal species. These early larvae are also, in both reciprocal crosses, unusually vigorous and seem to excel the larvae of either parent species in viability and rapidity of growth. It is important to note here that *F. heteroclitus* develops somewhat more rapidly than *F. diaphanus*, but that the developmental rates of both reciprocal crosses are more rapid than those of the respective egg species, and that in the case of the *F. diaphanus* egg hybrids this is very marked. These facts are entirely out of accord with the observations and statements of Moenkhaus, who claims that the invariable effect of the foreign sperm in fish hybrids is to retard development, never to accelerate it. He considers this universal retardation to be the result of some injury to either the egg or sperm substances of the zygote, akin to the well known hemolytic effects observed in experiments dealing with the transfusion of foreign blood. The hemolysis parallel fails to apply, however, in these cases and in others where no retardation occurs but where a pronounced acceleration is evident to any one who takes the trouble to make carefully controlled studies of comparative rates of development of pure and hybrid strains. In these crosses in which early acceleration has been observed it is conceivable that we are dealing with effects akin to those rejuvenating or stimulating effects so often noted when diverse strains of the same stock are crossed. Certain new combinations of morphological and physiological characters are more readily produced and occur more rapidly

than those normal to either strain or species. I am inclined to interpret the speeding up of the developmental process in these hybrids as the result of the introduction by the sperm of a foreign enzyme, which produces abnormally rapid dissociations in the egg materials, and in this way hastens the processes of metabolism and development. Whatever be the chemical explanation of the acceleration the fact remains that at a very early period, certainly long before the end of cleavage, the hybrid eggs are developing more rapidly than the pure bred ones. This can be detected without the use of any refinement of method and should be obvious at a very much earlier period if the methods previously used by the writer (Newman '10) were employed. If anyone has abundant time and patience he might readily demonstrate an acceleration in the first cleavages, but the writer has foresworn any further attempts of this character as involving labor incommensurate with the reward involved.

Next in point of developmental success are the crosses between the eggs either of *F. diaphanus* or of *F. heteroclitus* and the sperm of *F. majalis*. Although the hybrids herewith treated develop successfully and produce a fair percentage of vigorous larvae they go much more slowly than the controls. It is also to be noted that the hybrids between *F. heteroclitus* ♀ × *F. majalis* ♂ are much more viable than those between *F. diaphanus* ♀ × *F. majalis* ♂.

An interesting commentary upon the generalization of Möenkehaus that foreign sperm always has a deleterious influence upon the development of the egg, resulting in retardation of development, is to be had from a study of the chart including figures 9 to 20. Here the developmental rates of eggs of *F. diaphanus*, fertilized by three species of *Fundulus* sperm are shown in three parallel vertical columns, the middle column showing the control (pure *F. diaphanus*) that on the left *F. diaphanus* eggs fertilized with *F. majalis* sperm, and that on the right eggs of *F. diaphanus* fertilized with *F. heteroclitus* sperm. It will be readily noted that the effects of the two types of foreign sperm are of an exactly opposite character, the one producing marked retardation and the other equally marked acceleration. No theory depending

on the idea that the effects of foreign sperm are always injurious, resulting in retardation, can explain both of these equally obvious results. Another point of rather general import is also illustrated by this chart. It often happens in cases where hybrid strains go faster or slower for the first few days of development, that, in so far as the general external evidences of degrees of development may be relied on as criteria, hybrid and pure strains reach a point at which they are for a short time on a par. This is well illustrated by the figures showing the three strains of *F. diaphanus* eggs at the end of one week (figs. 18, 19, 20). After this time acceleration or retardation is again evident, usually up to the period of hatching. I am unable at present to offer any suggestion as to the conditions underlying this apparent interruption in the developmental rhythms of these strains.

In all cases in which hybrids successfully weather the period of gastrulation and enter upon the period of embryo formation, further success seems to be conditioned by the ability or inability to establish nutritive relations with the yolk. In cases where hybrids differentiate a circulatory system but fail to complete the assimilation of yolk, the difficulty is evidently due to lack of balance between the rate of embryonic differentiation and that of yolk digestion, as will be shown later. Although in the hybrids under discussion (*F. diaphanus* ♀ × *F. majalis* ♂ and *F. diaphanus* ♂ × *F. majalis* ♀) a great many of the embryos become abnormal for these reasons, it must not be forgotten that in every experiment a large proportion of them surmount the difficulties of yolk assimilation and produce normal vigorous larvae, some of which, at least in the *F. heteroclitus* egg hybrids, are more viable and grow faster than either of the pure bred strains. It is difficult to imagine what factors underlie this wide range of success of individual hybrids of the same parentage. It has been suggested that the best results are obtained when both germinal products are at the optimum state of maturity, and that if either one or the other germ cell be at all under-ripe or stale the result is sub-optimal development of varying degrees depending on the degree of over- or under-ripeness of egg or sperm. A more nearly probable explanation, how-

ever, is based on an almost diametrically opposite assumption and is suggested by a series of experiments which I performed with the idea of testing the effects upon development of freshness and staleness of the germinal products. Similar results were obtained several times, but sometimes what seemed to be the same treatment gave different results. Eggs of *F. heteroclitus* were fertilized with the sperm of *F. majalis* which had been kept alive for about twenty minutes in 45 per cent sea-water, a concentration that greatly prolongs the life of these spermatozoa. A very small percentage of eggs cleaved, but these developed normally and showed scarcely any trace of the paternal influence either in rate of development or in details of inheritance; while the control strain, from the same batch of eggs fertilized at the same time by more sperm obtained fresh from the same male, showed the usual retardation in development and the typical hybrid characters described here and subsequently. It might then be concluded that, when the sperm is so stale that it is barely able to initiate development in the egg, it plays a rôle equivalent to that of the reagents that produce artificial parthenogenesis, but is unable to take part in the differentiation of hereditary characters. According to this view, *the most active sperms might have the most deleterious effects upon the egg materials of another species and give rise to serious incompatibilities whose result is more or less pronounced abnormality, and cessation of development; while the sperms that have lost some of their vigor are less likely to disturb the developmental rhythm of the egg and thus more likely to give normal embryos.*

*b. Unsuccessful crosses*

It will have been noted that, although the reciprocal crosses produce viable larvae, the hybrids between *F. majalis* ♀ and *F. diaphanus* ♂ and those between *F. majalis* ♀ × *F. heteroclitus* ♂ *never produce larvae, since they never truly hatch. They* may live for weeks within the egg membrane but gradually succumb to anemia. The difficulty is almost certainly one involving retarded or partial yolk assimilation, since in the final stages

of development there is a large external yolk sac full of undigested yolk. In fish the development of the pericardium, heart and vitelline circulation are intimately associated, and under normal conditions the yolk sac diminishes in size at such a rate that it, together with the pericardium and heart, are simultaneously drawn into the body cavity of the embryo shortly after hatching. In these abnormal hybrids, however, the differentiation of the heart and pericardium is at first accelerated, while yolk digestion goes more slowly than in pure bred embryos. Since the three structures are intimately associated, this failure of the yolk sac to diminish causes the pericardium to enlarge and stretches the heart into a long straight tube which beats feebly but carries, in later stages at least, no blood. Thus the supply of nutriment is cut off and growth ceases. Various organs continue to differentiate even without an external food supply, so that we may have an embryo developed which, though much smaller than the normal, has reached a stage of advancement equivalent to that seen in young larvae of pure bred strains. In last analysis the stoppage of development seems to be conditioned by a lack of coördination between two processes, that of the differentiation of the protoplasmic materials, which is accelerated by certain agents brought by the foreign sperm, and that of yolk assimilation which fails to progress as rapidly as in normal eggs. The result is that the embryo gets to the point when heart pericardium and yolk sac should be taken into the body cavity and is prevented from so doing by the large mass of undigested yolk. The yolk of *F. majalis* is optically denser and of different color from that of the other species and it may well be that a specific enzyme carried by the sperm of the same species is necessary for its complete dissociation and assimilation.

When I say that these abnormal embryos, burdened as they are with a permanent yolk sac, never hatch, I do not mean that they may not be shaken or dissected out of their membranes. When Bancroft claims that these embryos occasionally hatch I judge that he means that they may sometimes lose the egg membrane. Real hatching, however, is brought about by well marked violent struggling of the larva, and no such hatching struggles

have been observed in any of the numerous experiments dealing with *F. majalis* egg hybrids that have been under my observation during the last eight years. Moenkhaus, moreover, agrees with me that these hybrids never hatch.

*c. Inter-generic crosses*

It is possible to produce six crosses between *Cyprinodon* and the three species of *Fundulus*, as shown in tables 5 to 10. In no case is the cross successful in the sense that a larva or even an advanced embryo is produced. None of the hybrids go far enough to show specific characters, but all go through the cleavage period more or less normally and some develop through the period of gastrulation and begin to show embryonic differentiation.

The six crosses may be significantly arranged in the order of their success in development, as follows:

1. *Cyprinodon* ♀ × *F. diaphanus* ♂. A small percentage of the embryos go practically through the period of gastrulation and begin to show the rudiments of embryonic differentiation.

2. *Cyprinodon* ♀ × *F. heteroclitus* ♂. A large proportion of the eggs go through to the end of the period of cleavage in a nearly normal manner and a few advance to a stage in which the germ ring has covered about one-third of the yolk and a flat embryonic shield is developed. Here the stoppage occurs in the midst of the process of gastrulation and before the embryonic axis is established.

3. *Cyprinodon* ♀ × *F. majalis* ♂. A few embryos go through the cleavage period and show the beginnings and early steps in gastrulation, a well defined germ ring with flat embryonic shield being formed, as in figure 11.

4. *F. diaphanus* ♀ × *Cyprinodon* ♂. Many embryos go through the period of cleavage and a few of them form a germ ring but get no further, the most advanced condition being like that shown in figure 9.

5. *F. heteroclitus* ♀ × *Cyprinodon* ♂. Many embryos develop through the cleavage period but fail to begin gastrulation, as indicated by the absence of any trace of a germ ring.

6. *F. majalis* ♀ × *Cyprinodon* ♂. In two experiments performed at different times none of the eggs lived through the cleavage period but began to disintegrate during advanced cleavage stages.

It is clear that for some obscure reason all of the *Cyprinodon* egg hybrids succeed better than any of the three types of *Fundulus* egg hybrids. It may be that this difference in success between reciprocal crosses is due to differences in the sizes of eggs and that it will be found that greater success generally is associated with the smaller egg, less burdened with yolk. Whatever factors may be found to condition the differences in developmental success between reciprocal crosses we have at least the clear evidence of such differences and these facts militate against the idea that the degree of developmental success in teleost hybrids is simply a function of phylogenetic relationship. To make myself clear on this point let me review the facts concerning reciprocal crosses between *F. diaphanus* and *Cyprinodon*. The *Cyprinodon* egg hybrid succeeds in passing safely through the period of gastrulation and in making a considerable start at embryonic differentiation, while the *F. diaphanus* egg hybrid succeeds only in barely beginning gastrulation. There is a marked difference here in developmental success without any difference in phylogenetic relationship. There must then be some factor other than phylogenetic relationship at the basis of the difference between such reciprocal crosses.

It should be noted also that all of these six inter-generic crosses under discussion, though the parent species all belong to the same family, Poeciliidae, develop much less successfully than several crosses between species belonging to different orders, as for instance some of those cited by Moenkhaus. A cross between *Fundulus heteroclitus* of the order Haplomi and *Gasterosteus bispinosus* (stickleback) of the order Hemibranchiae, gives hybrids in which, although the germ ring is only one-third around the yolk, the "embryo is short and completely formed." Again a cross between *F. heteroclitus* of the order Haplomi and *Menidia notata* of the order Acanthocephali give hybrids in which the embryo is described as being sufficiently differentiated to show



optic vesicles. Numerous other cases of this sort are found in Moenkhaus's paper, while other crosses between these same pairs of orders give a much lower degree of success. Even Moenkhaus's own data, therefore, fail to support his claim as to the relation between kinship and developmental success.

*d. General considerations concerning the influence of foreign sperm on the development and inheritance of teleost hybrids*

After working in this field for a number of years, it now seems to me that we are dealing with two quite distinct types of phenomena, the first a matter primarily biochemical in character and the second a matter of genetics. When we are dealing with rates of development we are invading a field more closely related to chemistry than to heredity, but when we attend to the reappearance in offspring of the specific embryonic or larval characters of the parent species we are dealing with legitimate problems in heredity. In thus excluding from the realm of hereditary effects of foreign sperm those which have to do with mere rate of cleavage and later development, I realize that I am taking a position totally at variance with that to which I adhered with considerable tenacity for several years and which I maintained in earlier papers dealing with *Fundulus* hybrids. At the time when these papers were written the observations that most impressed me were those that had to do with the *rate of development* of hybrid embryos and the time of appearance of the various embryonic structures. In both reciprocal crosses between *F. heteroclitus* and *F. majalis* it happened that the rate of development of the hybrids was intermediate between that of the two parent species. This was true of the cleavage rate, rate of germing formation, time of appearance of first pigment and time of establishment of heart-beat and circulation. On this basis the conclusion was reached that, with regard to the great majority of characters studied, the blended rather than the alternative type of inheritance prevailed. Now, however, that I have many cases before me in which the rate of development of the hybrids bears no relation to that of the parents, I am convinced that

characters depending upon rate of development are not really matters of inheritance at all but matters primarily of physiological chemistry. For example, although *F. heteroclitus* develops at a higher rate of speed than *F. diaphanus*, both reciprocal crosses have a higher rate than the pure bred strains. There is no intermediate condition here and therefore no blended inheritance. Similarly, when we make reciprocal crosses between *Cyprinodon* and any species of *Fundulus* we find a marked retardation in developmental rate in both crosses, whereas if rate is a matter of inheritance we would expect the *Fundulus* egg hybrid to inherit from the *Cyprinodon* father a markedly accelerated rate. I am inclined to believe, on the basis of evidence now available, that it will be found generally true that *in crosses between very closely related species the rate of development will be accelerated, while those between distantly related species development will be retarded, but not necessarily in direct proportion to the heterogeneity of the cross.*

These are the considerations that have led me to modify my former views and to conclude that, *although foreign sperm may materially alter the rate of early development, it plays no rôle in the heredity of the organism until embryonic differentiation is well under way.*

#### INHERITANCE OF PIGMENT CHARACTERS IN *FUNDULUS* HYBRIDS

##### I. A STATEMENT CONCERNING PIGMENT CHARACTERS IN *FUNDULUS* SPECIES

The study of the inheritance of pigment characters in the various crosses is a complex and very difficult one, involving as it does a consideration of the development, time relations and distributions of several distinct types of chromatophores, each with its peculiar specific characters. In order to simplify the issue as far as possible I have endeavored in most cases to limit my descriptions to the definitive conditions of these cells as they appear in the young larvae or, in the cases of non-hatching hybrids, in embryos of a stage of differentiation equivalent to that of the pure bred larvae selected for study.

Pigment cells may be conveniently classed as 'body' and 'yolk' chromatophores. Four kinds concern us in this study: black body, black yolk, red body and red yolk. The red chromatophores differ specifically in form, size, color, number and distribution. They may readily be distinguished, even when the color with transmitted light may appear brown instead of red, by the fact that they have an opaque whitish appearance when seen by direct light. The black chromatophores range from dark brown to gray to black and differ specifically in the same respects as the red cells.

Bancroft ('12) has added very materially to our knowledge of the inheritance of pigment characters in teleost hybrids. Using the same two species of *Fundulus* that furnished the basis of my previous experiments he has carried his analysis of this phase of heredity much further than I did. In my new experiments I have had occasion to repeat much of this work of Bancroft's and find little to criticize concerning his observations. If I were to attempt a general criticism of the work I would be inclined to ask whether he has not allowed his tendency to find Mendelian dominance in all pigment characters to obscure his vision for the many evidences of incomplete dominance or blending. If one is on the lookout for cases of dominance and is not too particular about its purity one can find an abundance of examples of it; but if one is on the alert for intermediate conditions or mosaics of maternal and paternal units, he can find abundant examples of them in every hybrid. Whether or not any particular significance attaches to the more or less pure dominance of unit characters in  $F_1$  hybrids between Linnean species, is an open question that we need hardly discuss here. Doubtless, were these hybrids fertile and capable of intercrossing, the parental characters would segregate out in the  $F_2$  generation of hybrids. Since no one has been able to get a second generation of teleost hybrids and since there is already a considerable literature on the inheritance of  $F_1$  hybrids between teleosts, it seems worth while to try to clear up the situation and to remove existing discrepancies as far as possible. With this idea in view I have performed these additional breeding experiments and offer the following considerably

wider range of data on pigmentation derived from four additional *Fundulus* crosses.

Bancroft's table on page 156 gives in brief form a characterization of the pigment differences found by him in the embryos and larvae of pure bred *F. heteroclitus* and *F. majalis* together with their reciprocal crosses. For convenience I shall use this table as a starting point, modifying it where it seems to me to be necessary and adding to it where it does not provide for new characters seen in the new hybrid types. For the sake of brevity the often repeated word 'chromatophore' is abbreviated to 'c.'

## 2. TABULATION OF DATA ON PIGMENT INHERITANCE

TABLE 11

*Showing pigment characters in Fundulus species, based on Bancroft's table 1, emended and expanded*

NO.	<i>F. HETEROCITUS</i>	<i>F. MAJALEIS</i>	<i>F. DIAPHANUS</i>
1	Red yolk chromatophores large and abundant, much branched and bright red (fig. 31)	Red yolk c. much fewer than in <i>F. het.</i> , very small and inconspicuous with a few fine branches, bright red color (fig. 32)	Red yolk c. large and abundant, much like <i>F. het.</i> except that color is red-brown (fig. 30)
2	Black yolk c. large and of a general polygonal shape, processes usually wanting	Black yolk c. smaller and with more and larger branches than in <i>F. het.</i>	Black yolk c. larger and darker than <i>F. het.</i> , also rounder in form and with many short radiating branches
3	First crop of head c. black and few in number, appearing about four days after fertilization; an interval before second crop	First crop head c. absent	First crop of head c. brown, numerous, appearing about three days after fertilization; no interval before second crop, as in <i>F. het.</i>
4	Lateral line with one continuous line of red c. at or before hatching; an occasional red c. far out of line	Lateral line without red c.	Three rows of red-brown c. on sides of body and tail, the middle one on the lateral line
5	Lateral line with none or but few black c. upon hatching	Lateral line with about 50 black c. upon hatching	Lateral line with about 30 black c., alternating with red-brown c., upon hatching
6	When yolk c. first appear there are as many on side opposite embryo as on embryonic side	Yolk c. first appear at sides of embryo and are entirely absent from opposite side	Yolk c. first appear on side opposite embryo and are larger and more numerous there than elsewhere
7	First yolk c. appear before any head c., at about 3 days	First yolk c. appear at about 5 days	First yolk c. appear after first head c., about 4 days after fertilization
8	Red c. on head	No red c. on head	Red-brown c. on head
9	Much fusion of black c. on head and yolk (fig. 22)	No fusion of any c. (fig. 23)	No fusion of any c. (fig. 21)

TABLE 12

*Showing pigment characters in hybrids between F. diaphanus and F. heteroclitus*

NO.	F. DIAPHANUS ♀ X F. HETEROCLITUS ♂	F. HETEROCLITUS ♀ X F. DIAPHANUS ♂
1	Red yolk c. intermediate in color between the maternal red-brown and the paternal brick red (fig. 33)	Same as reciprocal cross, but a little smaller on the average (fig. 34)
2	Black yolk c. like F. diaphanus in size and branching, but show a tendency to fuse as in F. heteroclitus	Same as reciprocal cross except that the type of c. is again almost pure F. diaphanus and there is scarcely a trace of fusion
3	Both maternal and paternal types of black head c. present; the maternal type appears first at about 3 days, followed by the paternal type in about 4 days (the first crop of Bancroft); each type has the characteristics of the pure species (fig. 24)	All black head c. of the F. diaphanus type, but there is much fusion as in F. heteroclitus (fig. 25)
4	Three rows of red-brown c. on sides of body, intermediate in color between those of two species	Same as reciprocal cross except that there are fewer c. to the row, the body being shorter
5	Black yolk c. larger and darker on side opposite embryo than elsewhere, as in maternal species	Black yolk c. less numerous on side opposite embryo than elsewhere; this is not like either parent
6	Lateral line with number of black c. intermediate between two species	Same as reciprocal cross
7	First yolk c. appear as early as in F. heteroclitus; time not intermediate	Same as reciprocal cross
8	Red c. of head intermediate in color between two species	Same as reciprocal cross
9	Fusion in the F. heteroclitus type of black c., but the F. diaphanus type isolated (fig. 24)	All black c. of the F. diaphanus type and fused extensively (fig. 25)

TABLE 13

*Showing pigment characters in hybrids between F. diaphanus and F. majalis*

NO.	F. DIAPHANUS ♀ × F. MAJALIS ♂	F. MAJALIS ♀ × F. DIAPHANUS ♂
1	Red yolk c. distinctly intermediate between the two parental types, in color, size and degree of branching (fig. 35)	Same as reciprocal cross, except that the paternal tendency to branch is stronger in (fig. 36)
2	Black yolk c. much smaller and more branched than in F. diaphanus, but larger and less branched than in F. majalis; a striking case of blended inheritance	Black yolk c. nearly as large and conspicuous as in F. diaphanus, but there is a marked tendency to give off long processes as in F. majalis
3	Black head c. practically pure F. diaphanus in form and size, but they appear later than in maternal species. This cannot be explained as the result of two crops (fig. 26)	Black head c. intermediate in type between those of two parent species (fig. 27); time of appearance also strictly intermediate
4	Lateral line with three rows of red-brown c. as in F. diaphanus, color intermediate	Same as reciprocal cross; here is a case of almost pure dominance of a paternal character
5	Lateral line with number of black c. intermediate between two species	Same as reciprocal cross
6	Black yolk c. less numerous on side away from embryo than in F. diaphanus; character intermediate	Same as reciprocal cross
7	Time of appearance of first yolk c. strictly intermediate	Same as reciprocal cross
8	Red c. of head and lips as numerous or nearly so as in F. diaphanus, but color is brighter than in maternal species	Red c. of head and lips seemingly larger and more conspicuous than in dominant parent species, color intermediate; case of hyperdominance
9	No fusion of c., since fusion is not a character of either parent species	Same as reciprocal cross

*Pigment characters of hybrids between F. heteroclitus and F. majalis*

It should not be necessary to repeat the facts concerning these crosses since they form the subject-matter of Bancroft's paper. An examination of his table 1 will reveal the facts as they appeared to him. A reëxamination of the same data, however, has convinced me that there is much more blending or intermediate inheritance and less pure dominance than Bancroft leads us to expect. He lists the following characters which he believes to be dominant:

1. "The character—presence of many large red yolk chromatophores (F. heteroclitus condition) is dominant over the character—presence of few small yolk chromatophores (F. majalis character)." Yet in the table and elsewhere he states that in the hybrids these elements are fewer, smaller and less branched than in the dominant parent.

2. "The size and shape of the black yolk chromatophores of F. heteroclitus is dominant over the size and shape of these same cells in F. majalis." Yet in his table we learn that these cells are smaller and have many minute processes, showing that the F. majalis influence is evident in the matter of size and shape.

3. "The presence of a first crop of head chromatophores appearing before the majority of the head chromatophores (F. heteroclitus condition) is dominant over absence of this crop of head chromatophores (F. majalis condition)." I have been unable to convince myself of the validity of this character since in many cases I have observed that the first crop consists of one chromatophore, that two or more followed after a considerable interval, and that the others came on gradually so that I have been unable to decide what constituted the first crop. All that I have been able to say for certain is that the first few chromatophores seem to be somewhat larger and to take longer to develop than the subsequent ones. A similar situation exists in F. diaphanus and here there is no distinction between the earlier and later chromatophores. I still believe that the time of appearance of the first chromatophores in the hybrids is strictly an intermediate character, and as such not an example of dominance.



4. "The presence of red chromatophores along the lateral line at hatching time or shortly before it (*F. heteroclitus* condition) is dominant over the absence of red chromatophores at the same time (*F. majalis* condition)." This is, beyond question, a case of dominance based on the presence of a positive character in one species and its absence in another. Bancroft fails, however, to mention that in the hybrids these red lateral line chromatophores are smaller and fewer in number than in pure *F. heteroclitus*.

To this list of Bancroft's I might add what seem to me to be two other characters as dominant as those just given. These are:

5. The character--red chromatophores on the head (*F. heteroclitus* character) dominant over the absence of red chromatophores on the head (*F. majalis* character). These red chromatophores are, however, fewer in number and smaller in the hybrids than in pure *F. heteroclitus*.

6. The character--fusion of black chromatophores (*F. heteroclitus* condition) is dominant over absence of fusion (*F. majalis* condition). In the hybrids, however, fusion is never so complete as in pure *F. heteroclitus*.

It is then apparent that no pure dominance is to be found in these hybrids and that partial dominance occurs only in such cases as those cited, in which a character is present in one species and absent in another.

### 3. SUMMARY AND DISCUSSION OF DATA ON PIGMENT INHERITANCE

1. When a character is present in one species and entirely absent in the other, the hybrids between the two species exhibit dominance of a more or less perfect type. Examples of this type of inheritance are as follows:

a. The character--one row of lateral line red chromatophores of *F. heteroclitus* is dominant over the absence of lateral line chromatophores in *F. majalis*, but the individual chromatophores are smaller and less complexly branched in the hybrids than in *F. heteroclitus*.

b. The character—three rows of lateral red body chromatophores of *F. diaphanus* is dominant over the single row of *F. heteroclitus* and the total absence of red body chromatophores of *F. majalis*. But in hybrid between *F. majalis* ♀ × *F. diaphanus* ♂ these chromatophores though brighter in color than in *F. diaphanus* are darker than in *F. majalis* and are larger and more conspicuous than in either parent species. This is a case of what in a previous paper I called hyperdominance.

c. The character—red chromatophores of forebrain and lips of *F. diaphanus* and *F. heteroclitus* is dominant over its absence in *F. majalis*, but in all crosses in which *F. diaphanus* is used there is a blending between brown color of *F. diaphanus* and the bright red of the other two species.

d. The character—fusion of head chromatophores of *F. heteroclitus* is dominant over its absence in the other species, but the degree and extent of the fusion in the hybrids is always less than in the dominant parent.

2. When a character occurs in both parent species but differs in the degree in which its various attributes express themselves, two alternative modes of inheritance are seen in the hybrids; either there appears a mosaic of the two types, each nearly pure in detail, or each of the units shows an intermediate condition as to the degree in which the various specific attributes find expression. Examples of both of these modes of inheritance are cited as follows:

a. In hybrids between *F. diaphanus* and *F. heteroclitus* both specific types of black head chromatophore appear to be dominant and occur side by side—a case of *mosaic or particulate inheritance*. Each type behaves throughout development like the homologous parental type, for example the *F. heteroclitus* type fuses with its neighbors to form small masses, while the *F. diaphanus* type remain as isolated cells.

b. In crosses between *F. heteroclitus* and *F. majalis* there is a blending with respect to the details of both red and black chromatophores wherever these occur. In the hybrids of both reciprocal crosses the individual chromatophores are smaller and

more finely branched than in *F. heteroclitus* and larger and less finely branched than in *F. majalis*. This is typical *blended inheritance*.

c. In crosses between *F. diaphanus* and either of the other species the color of the red chromatophores of the hybrids is distinctly *intermediate* between that of the two parental species.

3. When numbers of integral variates are concerned the hybrids exhibit a number intermediate between those of the two parents; or when one parent has many of a given type of integral variate and the other has none the number in the hybrids is less than that in the parent showing the characters in question. The following examples illustrate this:

a. In both reciprocal crosses between *F. heteroclitus* and *F. majalis* the hybrids show an intermediate condition between the *F. heteroclitus* character, few or no lateral line black chromatophores, and the *F. majalis* character, many such cells.

b. In the cross between *F. majalis* ♀ × *F. diaphanus* ♂ there are fewer lateral red chromatophores to the row than in *F. diaphanus*, although there are no lateral red chromatophores in *F. majalis*.

c. In hybrids between *F. diaphanus* and *F. heteroclitus* the hybrids have fewer black head chromatophores of the *F. diaphanus* type than has pure *F. diaphanus* and fewer black head chromatophores of the *F. heteroclitus* type than has the pure *F. heteroclitus*, yet the combined number of the two types of chromatophores is, as nearly as I can determine, intermediate between the total numbers of the two parent species.

When, therefore, we analyze one set of inherited characters such as the pigment characters that we have just dealt with, we find practically all of the well-known modes of inheritance; that characterized by *dominance* or even *hyperdominance*; that commonly called *blended* or *intermediate* inheritance; that usually called *mosaic* or *particulate* inheritance; and that which is peculiar to integral variates and has sometimes been classed as a phase of intermediate or blended inheritance, but which seems to me to be *sui generis*.

It is still an open question whether all of these types could successfully be reduced to an orthodox Mendelian basis, were the unit factors involved all analyzed out. Personally I would not enjoy the prospect of untangling these ultimate inheritance units from the conglomeration that exists in the hybrids of the  $F_1$  generation. If such an analysis were possible at all it could be made only from data derived from  $F_2$  hybrids. Any attempt to proceed very far with such an analysis upon the  $F_1$  hybrids would seem to be pushing the factor hypothesis too hard, without being of any value to the subject of genetics, for the principle of *dominance* is at best of minor significance as compared with the principle of *segregation*. In the  $F_1$  generation of hybrids we may readily have pure dominance, blends, intermediates, mosaics or particulate mixtures without doing violence to the essentials of Mendelian inheritance, for the original unit factors, whether pure dominants, blends or mixtures, segregate out in the pure state in the next generation of hybrids, thus satisfying all of the demands of the theory.

This then is my conclusion: that the  $F_1$  generation of hybrids affords unfavorable material for the study of Mendelian inheritance and that unless we can interbreed the  $F_1$  hybrids we can make little progress in the analysis of the inheritance factors. Yet certain facts of much importance from the standpoint of developmental physiology may be determined by means of teleost hybrid material, some of which we have discussed and shall more fully discuss in this paper. I still feel as I did when I wrote my earlier papers that for the purpose of Mendelian breeding work teleosts are unfruitful material, but that they throw a great deal of light on the process of heredity. In this respect teleost material is no better and no worse than echinoderm material, which has been so extensively exploited.

## GENERAL SUMMARY OF CONCLUSIONS

*1. The nature of the influence of the spermatozoön on cleavage and development*

The new evidence here offered tends to support the position that the rate of cleavage is largely a function of egg size and egg constitution and is inherited only in this restricted sense, just as certain factors of the environment may be said to be inherited, because the organism finds them ready made for its use. Foreign sperm, when introduced into the egg, may accelerate or retard cleavage or later development, but in so doing is not exercising an hereditary function, for there are cases in which the sperm of a more rapidly developing species retards development and others in which the sperm of a more slowly developing, but closely related species, hastens development. This is surely not heredity. I would like to suggest that the influence exerted by foreign sperm upon the rate of development is chemical and mechanical in nature and that it is only when the sperm components of the zygote begin to coöperate with the egg components in bringing about the differentiation of specific characters, that the sperm begins to play its rôle as a factor in heredity. It is doubtless during the process of gastrulation that the first steps in differentiation take place and it is very interesting to note that in so many heterogenic crosses the developmental stoppages occur at the onset of or during the process of gastrulation. The conclusion would seem to be obvious then that any teleost spermatozoön may play a rôle in cleavage equivalent to that of agents that are successful in artificial parthenogenesis, but that only certain special kinds of sperm, that can successfully coöperate with the egg nucleus in its hereditary activities, are capable of working out a complete ontogeny.

### 2. *Harmful versus beneficial effects of foreign sperm*

The effects of foreign sperm are not always deleterious nor is it true that cross fertilization always results in a retardation or interruption of early development, as Moenkhaus maintains. In fact the sperm of a closely related species may, and actually does in three out of six *Fundulus* crosses, definitely accelerate development from the very beginning, and produces a hybrid larva more viable and capable of growing faster than those of either parent species. The idea of Moenkhaus then, that foreign sperm plays a rôle equivalent to that of foreign blood in transfusion experiments, is out of accord with these facts, for there is nothing equivalent to the injurious hemolytic effects of foreign blood in crosses where the sperms of closely related species are used. On the contrary, an effect is produced akin to the well-known stimulating effects of crossbreeding strains in practical animal husbandry.

### 3. *Phylogenetic relationship of parent species and developmental success of hybrids*

The idea expressed by Moenkhaus that success in the development of teleost crosses is a function of the phylogenetic relationship of the parent species breaks down in view of two sets of facts:

a. There is a marked difference between the developmental success of reciprocal hybrids. The parents are the same in both, hence factors other than that of phylogenetic relationship must underlie this different degree of success in development.

b. The data given in this paper, and by Moenkhaus himself, prove that success in development of hybrids produced by crossing different orders of teleosts is not seldom greater than that attained by hybrids between different families of the same order, or even between different genera of the same family.

Kammerer endeavors to show that success in crossing depends on similarity of habitat, but in these experiments of mine a fresh water and a marine species give the most normal development, while two marine species give unsuccessful crosses. The

problem as to the factors involved in the relative developmental success of reciprocal crosses, and of hybrids between various species, is unsolved.

*4. Modes of inheritance in hybrids and the Mendelian hypotheses of dominance and segregation*

It has been shown that structural and physiological characters in the  $F_1$  generation of teleost hybrids show exclusive, blended and particulate inheritance. There is seldom if ever pure dominance and there is frequently an almost perfect intermediate condition between parental types, especially in the case of pigment characters. In some cases there is a sort of regional dominance of one parental type in one area and a similar regional dominance of the other parental type in another area; or else the two types may exist side by side in quite intimate contact with each other. These mosaics and mixtures are typical examples of what we have come to call particulate inheritance. In the case of integral variates the hybrids exhibit numbers of variates intermediate between those of the parents. So we find practically all known types of inheritance in connection with pigment characters alone, and would also find a similar condition for any other group of characters studied equally intensively. The discovery of the prevalence of intermediates and blends in the  $F_1$  generation does no violence to the fundamental principles of Mendelian inheritance, for it is practically certain that these characters that seem to blend in the first generation of hybrids would segregate out in typical fashion in subsequent hybrid generations, were it possible to interbreed the  $F_1$  hybrids. We have nothing to offer, therefore, that need be interpreted as out of accord with recent Mendelian work, although previous work dealing with the same kind of data has been cited not infrequently as furnishing examples of non-Mendelian inheritance.

*5. Inheritance in reciprocal crosses*

It must be obvious to the reader that in these and other teleost crosses reciprocal hybrids differ materially in many respects. It has been shown repeatedly that in the majority of cases where in the hybrid a character appears as a blend between that of the two parents, it approximates more closely the maternal than the paternal condition. There are, however, exceptions in which the paternal influence predominates. Again we have cases in which in one reciprocal cross a given character will be almost entirely like that of one parent, whereas in the other reciprocal cross we may have a mosaic or a blend of the two parental conditions. There are also cases in which the male influence predominates in both reciprocal crosses, but is less pronounced in one cross than in the other. The facts do not admit of being classed in the category of sex-linked characters, for all hybrids of a strain, which presumably consists of equal numbers of males and females, show the same conditions. Nor is the inequality of reciprocal hybrids to be explained by assuming that there may be a partial elimination of paternal chromatin, as was the case in Baltzer's echinoderm hybrids, for we have abundant evidence that in all of these *Fundulus* crosses the paternal chromatin functions normally. There is evidently a problem here the solution of which demands a much larger range of data than has as yet been obtained. I am for my own amusement positing a number of tentative hypotheses that may or may not stand the test of experiment, but shall not risk at present a premature display of a tendency to jump to conclusions. As it stands the problems involved in the differences between reciprocal crosses are unsolved.



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## PLATE 1

## EXPLANATION OF FIGURES

1 to 8 represent the four species of fish used in the present experiment. Both males and females are shown life sized.

- 1 *Fundulus heteroclitus* adult female.
- 2 *Fundulus heteroclitus* adult male.
- 3 *Fundulus majalis* adult female.
- 4 *Fundulus majalis* adult male.

MODES OF INHERITANCE IN TELLOST HYBRIDS  
H. H. NEWMAN

PLATE I



1



2



3



4

## PLATE 2

### EXPLANATION OF FIGURES

- 5 *Fundulus diaphanus* adult female.
- 6 *Fundulus diaphanus* adult male.
- 7 *Cyprinodon variegatus* adult female.
- 8 *Cyprinodon variegatus* adult male.



5



6



7



8

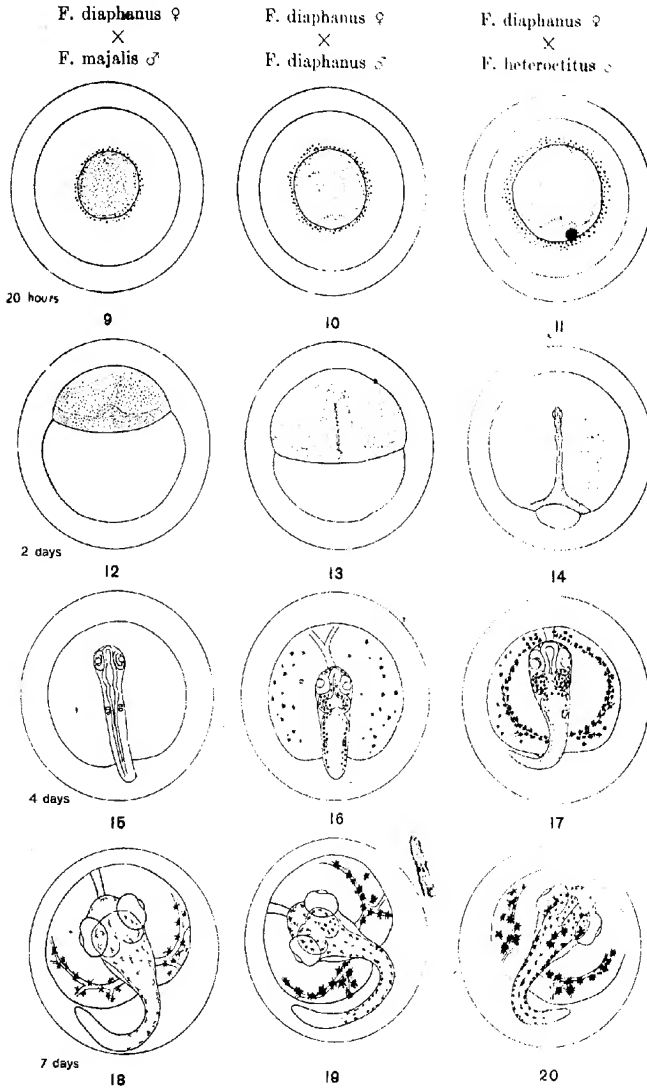
### PLATE 3

#### EXPLANATION OF FIGURES

9 to 20 form a developmental chart, showing the comparative rates of development of embryos of pure *E. diaphanus* (middle column) and the two types of *E. diaphanus* egg hybrids (that from *E. majalis* sperm on the left and that from *E. heteroclitus* sperm on the right). Note that *E. majalis* sperm retards early development and that *E. heteroclitus* sperm as markedly accelerates it.

MODES OF INHERITANCE IN TELEOST HYBRIDS  
H. H. NEWMAN

PLATE 3



## PLATE 4

### EXPLANATION OF FIGURES

21 to 29 Show the various types of black head chromatophores found in young larvae, at hatching or at a period equivalent to this in non-hatching embryos. All camera drawings under same magnification  $\times 100$ ).

21 The isolated stellate type of *F. diaphanus*.

22 The polygonal, unbranched, fusing type of *F. heteroclitus*.

23 The radiate type with tooth-like branches on the rays, characteristic of the hatched larvae of *F. majalis*.

24 A typical group of head chromatophores in the hybrid larva from egg of *F. diaphanus* and sperm of *F. heteroclitus*. Note the mosaic of isolated, stellate *F. diaphanus* cells and the fused groups of polygonal *F. heteroclitus* cells.

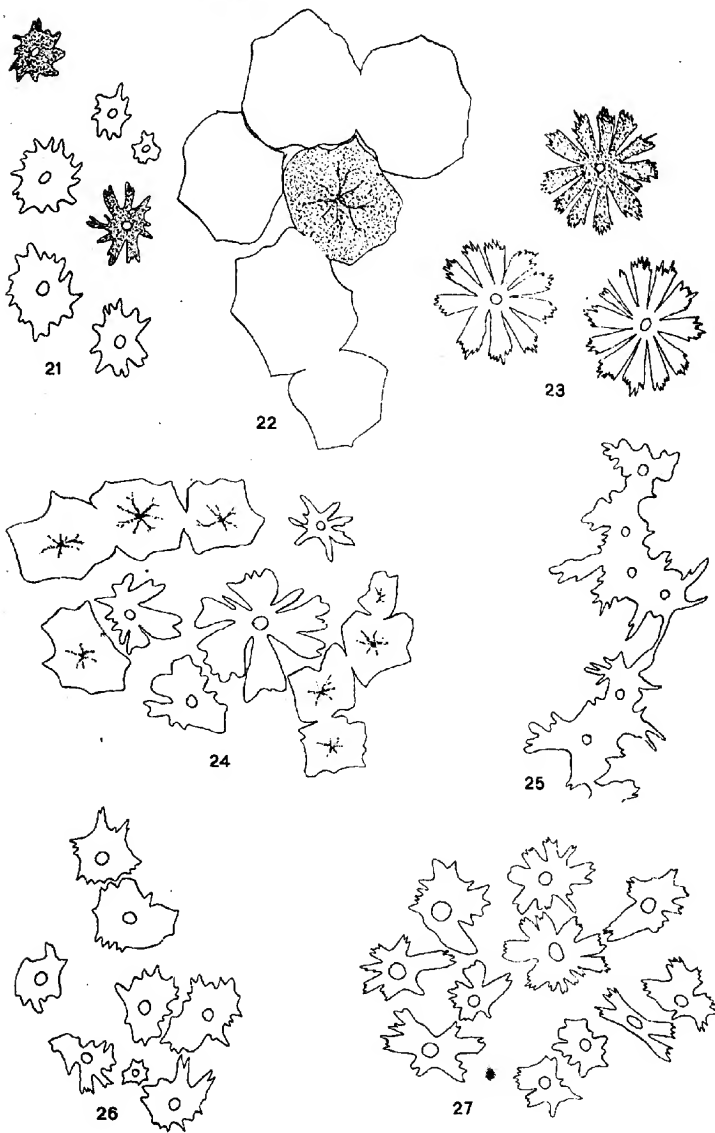
25 A typical group of head chromatophores of the reciprocal hybrid larva, from *F. heteroclitus* egg and *F. diaphanus* sperm. Here we find only the stellate cells of *F. diaphanus*, but they show the *F. heteroclitus* character of fusion.

26 A typical group of head chromatophores from the hybrid larva from *F. majalis* egg and *F. diaphanus* sperm. The paternal type is almost entirely dominant.

27 A typical group of head chromatophores from the reciprocal hybrid from *F. diaphanus* egg and *F. majalis* sperm. The cells are intermediate in size and form between those of the parent species.

28 A typical group of head chromatophores in hybrid larva from *F. heteroclitus* egg and *F. majalis* sperm. The *F. heteroclitus* polygonal fused type predominate, but in certain regions a modified *F. majalis* type of cell is fairly abundant.

29 Group of head chromatophores in hybrid from *F. majalis* egg and *F. heteroclitus* sperm, showing dominance of the paternal type.





## PLATE 5

### EXPLANATION OF FIGURES

30 to 38 Represent approximately the form and color of the chromatophores in pure and hybrid strains.

30 Red-brown cell of pure *F. diaphanus*, showing profuse branching.

31 Brick red cell of pure *F. heteroclitus*, showing large size and many branches.

32 Brick red, but very small inconspicuous cell of *F. majalis*, showing hair-like branches.

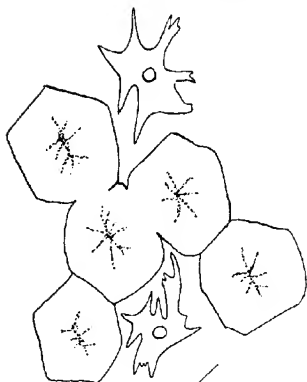
33 Type of red chromatophore found in hybrid larvae from *F. diaphanus* egg and *F. heteroclitus* sperm. These cells are less branched than in *F. diaphanus* and are intermediate in color between red-brown and brick red.

34 Type of red yolk chromatophore found in hybrids from *F. diaphanus* sperm and *F. heteroclitus* egg. Branches are shorter than in reciprocal cross; color same as reciprocal cross.

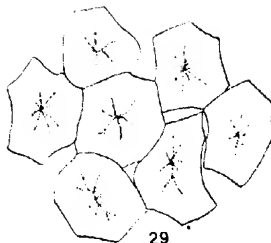
35 Type of red chromatophore in hybrids from *F. diaphanus* egg and *F. majalis* sperm. Strictly intermediate in every particular.

36 Type of red chromatophore in hybrids from *F. majalis* egg and *F. diaphanus* sperm. Like reciprocal cross except that cells are more branched.

37 and 38 Types of red chromatophore in the two reciprocal crosses between *F. heteroclitus* and *F. majalis*. Fig. 37 the *F. heteroclitus* egg hybrid, figure 38 *F. majalis* egg hybrid. In both cases the intermediate condition is evident.



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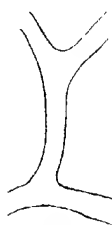
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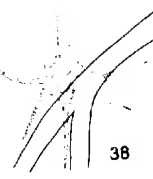
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## THE BEHAVIOR OF THE CHROMATIN IN HYBRIDS BETWEEN FUNDULUS AND CTENOLABRUS

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THIRTY-SIX FIGURES (FIVE PLATES)

The following investigation was begun in the summer of 1911 at the suggestion of Dr. Kellicott of Goucher College, and the material was collected at Woods Hole in the summers of 1912 and 1913. I am indebted not only to Dr. Kellicott for the suggestion, but also to the members of the Zoological Faculty of Yale University for the privileges extended to me in the Yale laboratories, and for their advice and encouragement while I was following out the problem.

*Fundulus heteroclitus* and the cunner, *Ctenolabrus adspersus*, are common in the waters about Woods Hole. The spawning season for both kinds of fish is approximately the same, extending from the last of May till the first of July. The eggs are easy to get from both species, and the sperm of *Ctenolabrus* can be easily expressed. The male *Fundulus* is more difficult to strip and it is best to tease out the testis in a watch-glass, add a few drops of water, and use this fluid to fertilize the eggs. The *Fundulus* eggs can be obtained at any time of day, and from females that have been some days in the laboratory; but the *Ctenolabrus* eggs are not good if taken from a female that has been in the aquarium over night. As the eggs do not ripen till late in the afternoon, it is best to have a fresh supply of *Ctenolabrus* brought in not earlier than 3 p.m. A few males will probable live over night, and their milt can be used for *Fundulus* eggs the next morning.

The usual precautions were taken to prevent normal fertilization of the eggs; that is, fertilization by the sperm of their

own species. The pipettes were not only carefully washed, but were marked besides, so that the one used for *Fundulus* sperm was never used for unfertilized or hybrid *Fundulus* eggs. Dishes were carefully washed and dried, and a control lot of unfertilized eggs from the same lot as the hybrids was always kept. No eggs were found in any of these control lots that had divided.

The eggs of *Fundulus* have a diameter of about 2 mm., while those of *Ctenolabrus* are about 1 mm. in diameter. Both kinds were handled as follows: they were expressed into a finger-bowl containing a small amount of water. A number were taken out for two controls, one of which was normally fertilized and the other left unfertilized. The rest of the eggs were fertilized with the foreign sperm, and more water was added to all three lots to keep them fresh.

The eggs were preserved in series at varying intervals, the fluids used being Gilson's, Bouin's, and Perenyi's for the *Fundulus* eggs and Flemming's strong mixture and Perenyi's fluid for the *Ctenolabrus* eggs. The method of embedding and sectioning used by Moenkhaus on *Fundulus* eggs worked very well for those preserved in Bouin's fluid. After the tough outer membrane had been removed, the eggs were embedded with the blastodisc uppermost, and sections 8 to 10  $\mu$  in thickness were cut horizontally through the blastodisc. The eggs preserved in Gilson's and Perenyi's fluids were differently treated, as a touch of the needle removes the blastodisc from the yolk, and it is best to embed the former alone. The *Ctenolabrus* eggs are more troublesome. They are too small to be easily embedded in a definite position, so the sections must be cut at random. Moreover, those preserved in Flemming's mixture are so dark that it is necessary to bleach them. I used Mayer's method, bleaching the sections on the slide, but found that sections so treated did not take the stain well. The stains used were Heidenhain's hematoxylin and iron alum, and Ehrlich's hematoxylin counterstained with Congo red. The latter is more satisfactory, as there are granules in the cytoplasm of the *Fundulus* egg which are stained by the iron hematoxylin method and may be confused with small chro-

mosomes. With Ehrlich's hematoxylin, however, no mistake can be made, as it is only the chromatic material that takes the deep purple stain, all the rest of the egg being stained red.

No records were made of the percentage of eggs dividing in the hybrid cultures, so that only the most general statements can be made on this point. The *Fundulus* eggs are fertilized readily with the *Ctenolabrus* sperm and it is estimated that about half of those in a hybrid culture go through cleavage. Division was much rarer in hybrid *Ctenolabrus* eggs, not more than 10 per cent of them being fertilized. The head of the *Fundulus* sperm is considerably larger than that of the *Ctenolabrus* sperm (figs. 35 and 36) and this may account for the greater ease with which the  $F \varnothing \times C \sigma$  cross is made.

Polyspermy is apparently rare. Only one tetraster was found in the preserved *Fundulus* material, and one case is recorded of a 4-cell stage in a culture in which the other eggs were in the 2-cell stage. This was probably a dispermic egg, dividing at once into four cells. There was no evidence of polyspermy in the *Ctenolabrus* eggs, but all the evidence from these eggs is poor, and polyspermy may possibly occur in them.

In neither cross did I succeed in raising the hybrids to a late stage of development. Loeb has raised hybrids from the same two species of fish, but it is necessary to put only three or four eggs into a single dish, and take particular care to change the water frequently. Even then the proportion of late stages is small, so that to have satisfactory results one must fertilize large numbers of eggs daily. With the limited space at my command, I was unable to use this method. The conditions under which I kept the eggs were perfectly satisfactory for the normally fertilized ones, but not for the less hardy hybrids. The *Fundulus* eggs died about twenty-four hours after fertilization, at the time when the embryo has begun to form and the germ-ring has covered about half of the egg. The *Ctenolabrus* eggs also died within twenty-four hours; but they had developed further in that time than the *Fundulus* hybrids, the natural rate of development being more rapid for *Ctenolabrus* than for *Fundulus*.

They reached a stage in which the embryo was fairly well formed and the germ-ring had gone two-thirds of the way around the egg.

The cytology of hybrids has attracted considerable attention in recent years. In 1904 W. J. Moenkhaus made a study of the hybrids between *Fundulus heteroclitus* and *Menidia notata*, which is of particular interest in the present connection. In the F. ♀ × M. ♂ cross, Moenkhaus finds that about 50 per cent of the eggs are dispermic, while in the reciprocal cross there are only a few which are poly- or dispermic. He did not succeed in keeping the hybrid larvae alive more than two or three days, and therefore made no study of their heredity. The most important part of the paper is the study of the germ-nuclei and the chromatin in the early cleavages. Moenkhaus says that the pronuclei fuse completely before the first cleavage, but he gives no figures to demonstrate that fusion. He passes at once from a figure which shows the pronuclei closely apposed—as he says, “about to fuse” to the metaphase of the first cleavage spindle. In this stage and in the anaphase, he finds two morphologically distinct kinds of chromosomes, each of which can be traced to one parent of the cross. His figures show the two types very plainly, and he traces these types as far as the late cleavage stage. At first the chromosomes are bilaterally grouped, that is, the paternal and maternal chromosomes are not mixed on the spindle, but in the third cleavage he finds this grouping destroyed, and as cleavage progresses the two types are completely mingled, though still persisting.

The work of D. H. Tennent ('08) on the eggs of *Toxopneustes* and *Arbacia* fertilized with *Moiria* sperm shows somewhat similar results. He finds that the pronuclei fuse completely; but in the Arb. ♀ × M. ♂ cross in which the two types of chromosomes are morphologically distinct, they are mingled, not grouped on the first cleavage spindle.

The paper of Jacques Loeb ('12) on heredity in heterogeneous hybrids forms a complement to the work of Moenkhaus, as it discusses the inheritance of maternal characters in hybrids between *Fundulus* and *Menidia*, *Ctenolabrus*, and *Stenotomus*.

without taking up the cytology of such eggs. Loeb kept these hybrids alive a month or more, and they formed the heart, blood-vessels, eyes, and fins, though they never hatched. He believes that the sperm in these cases acts as a parthenogenetic agent, simply removing from the egg obstacles to its division. The hybrids are therefore 'pure breeds' and the abnormalities which occur are due to the interference with the normal chemical reactions caused by the introduction of a foreign sperm. These abnormalities are, he says, "in no sense hybrid characters" and he caused similar abnormalities by keeping normally fertilized *Fundulus* eggs in sealed Erlenmeyer flasks containing 50 cc. seawater + 2 cc. 0.01 per cent NaCN. In two instances, however, he finds in *Menidia* eggs fertilized with *Fundulus* sperm, red chromatophores such as are common in *Fundulus* eggs but are not present in normal *Menidia* eggs. This seems like genuine inheritance of a paternal character—the only evidence of such inheritance he has found in these experiments.

Many writers in discussing the purely maternal character of heterogeneous hybrids explain this one-sided inheritance by an elimination of the paternal chromatin which may take place in the early cleavages. Among these are Kupelwieser ('09), Baltzer ('10) and Tennent ('08 and '12). Kupelwieser fertilized sea-urchin eggs with mollusk sperm and found that although the eggs divided, the sperm had no part in this division. It lies at the side or at one pole of the spindle and probably degenerates in the 2-cell stage. He did not, at this time, get larvae old enough to show whether the characters were purely maternal or not. Later ('12) he fertilized *Echinus* eggs with the sperm of an annelid, and found that although the pronuclei almost always fuse before the first cleavage, the behavior of the male chromatin in the first cleavage spindle is always abnormal, and it is not included in the daughter-nuclei of the 2-cell stage. He traces the abnormalities of the larvae, which are maternal in character, to mechanical disturbances of the maternal chromatin caused by the undivided mass of male chromatin on the spindle.

Baltzer made various crosses between *Echinus*, *Strongylocentrotus*, *Sphaerechinus*, *Arbacia*, and *Antedon*, and found in many



cases that a definite number of chromosomes which he was often able to identify as paternal in origin were eliminated in the early cleavages. When this was the case, the larvae showed only maternal characters. When the male chromatin was retained the larvae were intermediate in character, except in the cases of *Echinus* ♀ × *Antedon* ♂ and Strong. ♀ × *Antedon* ♂ where there is no elimination of chromatin. In this case, nevertheless, purely maternal hybrids result. Tennent ('12) found much the same condition in hybrids between *Hipponoë* and *Toxopneustes*. In *Tox.* ♀ × *Hip.* ♂ there is no elimination of chromatin, and the male dominates; while in the *Hip.* ♀ × *Tox.* ♂ cross, in which the maternal characters dominate there is elimination of a certain amount of chromatin which, although it cannot be traced definitely to either parent, is presumably of paternal origin. In crosses between *Arbacia* and *Toxopneustes* ('12 b) he finds that some chromatin of female origin may also be eliminated.

In the hybrids with which the present paper is concerned, we find a condition differing somewhat from any of these, though naturally most like that described by Moenkhaus for the *Fundulus* × *Menidia* cross. As has been said, it is necessary to draw all conclusions from the *Fundulus* eggs as the material obtained from the reciprocal cross was unsuitable for study. The first stage of interest in the hybridized eggs of *Fundulus* is that represented in figures 1, 4 and 5, where the germ-nuclei are closely apposed, and the aster has divided. In figures 4 and 5 the asters lie at opposite poles of the pair of nuclei, while in figure 1 they have not yet taken that position. It will be noticed also that in one case the nuclei lie side by side, and in the other end to end. In many cases one can see a difference between the two nuclei in the density of the reticulum, but this is not universal, and it is impossible to say with any degree of certainty which is the male and which is the female. A study of the normal *Fundulus* egg shows that this failure of the germ-nuclei to unite is peculiar to the hybrids, and that the stage represented in figures 1, 4 and 5 is in fact that which immediately precedes the formation of the first cleavage spindle. The condition of the normally fertilized eggs is shown in figure 3. As long as the two

nuclei are separate the aster is undivided (see fig. 2) and it is only after complete fusion of the nuclei has taken place, as will be seen in figure 3, that the asters assume a polar position like that shown in figures 4 and 5.

The stage which immediately follows shows still more plainly that the pronuclei do not fuse in these hybrids. This stage is shown in figure 6, and the condition represented there has been found in all the hybrids of this stage. Figure 7 shows a normally fertilized *Fundulus* egg in a corresponding stage, and the difference between the two is obvious. In the hybrid there are two dark masses lying in the spindle, in which masses the chromosomes can be seen. In the normal egg a single mass of the same nature is present, evidently the fusion nucleus. Taken in connection with the total absence of single nucleus stages in the hybrid, the evidence shows that although the germ-nuclei fuse in the normally fertilized *Fundulus* egg, they do not do so when the egg is fertilized with *Ctenolabrus* sperm.

The chromosomes of *Fundulus* are much larger than those of *Ctenolabrus*. They vary somewhat in shape according to their stage of development, but they are always easily to be distinguished from the small round *Ctenolabrus* chromosomes. In the metaphase the *Fundulus* chromosomes are long, but not straight. They have usually a thickening at one end. This can be seen in figure 10 which shows a few chromosomes from a normal metaphase group. Figure 15 shows the *Fundulus* chromosomes in the anaphase, where they have lengthened and straightened. Figure 9 shows a spindle from a normally fertilized *Ctenolabrus* egg, with the typical round *Ctenolabrus* chromosomes.

In the hybridized *Fundulus* egg, these two types of chromosomes are easily seen in the first cleavage spindle. In the metaphase, indeed, the chromosomes lie so close together that the two types can hardly be distinguished, but as soon as they begin to draw apart one sees the difference very plainly. Of course in the study of these stages, sections should be selected in which all the chromosomes are included. Otherwise, small pieces of the *Fundulus* chromosomes might be confused with the *Ctenolabrus* chromosomes. Figure 11 shows a spindle in which the

chromosomes have begun to separate. In this case the types are grouped, the male chromosomes lying on one side and the female on the other side of the spindle; but this is not always the case. Figure 8 shows chromosomes from a similar stage in which the two types are mingled.

In the later anaphase the difference is still more evident. Here I find, as did Moenkhaus, that the foreign chromosomes sometimes lag behind the normal ones in going to the poles. Figures 12 and 17 show the anaphase of the first cleavage in two hybrid eggs, while figures 13 and 18 give the same stage for the normal eggs. The difference between the hybrid and normal eggs is distinct and constant except in the case shown in figure 19. This is drawn from an egg found on a slide that was supposed to have only normally fertilized eggs on it; but it is more reasonable to suppose that, by a mistake in the handling of the preserved material, a hybrid egg was included among the normal ones than that one normal egg should present an appearance so like the hybrids and so different from all the other normal eggs. Figures 14 and 16 show the chromosomes from the metaphase of the first cleavage of two hybrid eggs, drawn with a magnification of 2500 diameters. They should be compared with the corresponding stage in a normal egg which is represented in figure 15. Even in the smaller-scale drawings, the difference between the two chromosome groups is apparent, but in the figures drawn with the higher magnification the distinctions between two types are particularly apparent.

The telophase of the first cleavage in these hybrids is very much like the corresponding stage in the normal egg. The vesicles in some cases fuse a little more slowly as the foreign chromosomes have lagged a little in going to the poles; but the difference is slight, and by the time the asters have separated and come to a polar position preparatory to the second cleavage, the nuclei of hybrids and normals cannot be distinguished from each other. In both cases one can see traces of the vesicles in the nuclear reticulum; and this is the case in the resting nuclei of both hybrid and normal eggs throughout development. Figure 20 shows the telophase of the first cleavage of a normal egg, and

figure 21 the same stage for the hybrid; while figures 22 and 23 represent the nuclei of the completed 2-cell stage in hybrid and normal eggs respectively.

Figure 24 represents the prophase of the second cleavage in a hybrid *Fundulus* egg. It will be seen that there is here, as in a normal egg, a single mass of chromatic material on the spindle quite different from the double mass formed by the ununited germ-nuclei before the first cleavage. This is added proof that in the nuclei of the 2-cell stage, the paternal and maternal chromatin is mingled. That the types of chromosomes do not, however, lose their identity is shown by the fact that in the later stages of the second cleavage they appear as distinctly as they did in the first cleavage (fig. 25). Throughout the later cleavage, moreover, the resting nuclei and prophases of hybrid eggs are indistinguishable from those of the normal ones, while the hybrids continue to show the two types of chromosomes in the anaphase stages. Figures 27 and 28 and figures 29, 30, 31 and 32 illustrate this fact. Figure 27 is of a fourth cleavage spindle and figure 28 of a sixth, while the others are from 12-hour stages.

With such figures as these last ones before us we should hardly look for any considerable elimination of chromatin in the early stages, and in fact there is no evidence that the paternal chromatin is thrown out in these hybrids. Figure 26 shows the prophase of the fourth cleavage in a hybrid, and in this a small mass of chromatin is found outside the spindle, but there is no evidence that this is of paternal origin. This is the only case of such abnormality I have found in the early cleavages, and the evidence of the later stages leads one to think that the paternal chromatin is generally retained and divides normally.

The first appearance of abnormality that can be called general is in the 12-hour stage. Here we find often, in the middle of the blastoderm, large cells with irregular nuclei, in which there has evidently been a failure to divide normally. Figure 34 shows such a cell, and figure 33 two more, with a few normal cells drawn for comparison. But even here we cannot say that

there is certainly elimination of paternal chromatin; for in another part of the same egg in which these abnormal cells occur, spindles are to be found like those represented in figures 29 to 32, in which the *Ctenolabrus* chromosomes are present. The disturbance is undoubtedly a result of the fertilization with the foreign sperm—we find no such condition in normally fertilized eggs—but we cannot say that it concerns the paternal chromatin alone. It seems, rather, to involve the whole cell, and is probably the cause of the death of many of the embryos and the abnormalities of those that survive. Where the cells continue to divide normally they retain the two types of chromosomes.

It will be seen that the cytological study of these eggs hardly supports Loeb's theory that the function of the sperm in such hybrids is merely that of a parthenogenetic agent. Nor do the conditions agree with those found in Echinoderm hybrids, with the exception of those between the *Echinus* and *Strongylocentrotus* females and the *Antedon* male. Although up to the 24-hour stage the larvae show no signs of paternal inheritance, it would be hardly safe to say that they were purely maternal in character, if it were not for Loeb's description of later stages of hybrids between these same two species of fish. As he, however, found no paternal characters in these larvae at any time, it must be concluded that the *Fundulus* egg fertilized with the sperm of *Ctenolabrus* gives purely maternal larvae without elimination of the foreign chromatin.

#### SUMMARY

1. In the cross *Fundulus* ♀ × *Ctenolabrus* ♂ the germ-nuclei are closely applied to each other, but form chromosomes for the first division without having fused.

2. The chromosomes of the first cleavage spindle are of two types, which correspond respectively to those seen in the normally fertilized eggs of the two species.

3. Although the foreign chromosomes lag somewhat behind the others in going to the poles, they are all finally included in the daughter-nuclei of the 2-cell stage.

4. The two types of chromosomes reappear in the spindle of the second cleavage, and can be distinguished throughout the development, which was followed as far as the 12-hour stage.

5. In the 12-hour stage large cells with irregular nuclei are found, which represent the beginning of disturbances later shown by abnormality or death of the larvae.

6. There is no evidence of elimination of the paternal chromatin at any stage.

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After the present article went to press a paper by Günther and Paula Hertwig on teleost hybrids appeared in the Archiv für Mikroskopische Anatomie (Bd. 84, Heft 2), the results of which agree in general with the above conclusions, though there is no case exactly parallel to that of *Fundulus* ♀ × *Ctenolabrus* ♂. The following points of agreement may be mentioned. The authors found no case of elimination of chromatin, and in most cases the paternal and maternal chromatin united, giving a perfectly normal cleavage. In all but one of the heterogeneous crosses, the larvae died in the blastula stage, or when the embryo was just beginning to form, and at this time the only cytological abnormality was the unusually large size of the nuclei. The case of *Ctenolabrus* ♀ × *Box* ♂, however, is different from their other heterogeneous crosses, for in this case the larvae were very nearly normal. They were not, however, parthenogenetic larvae, as can be shown from the size of their nuclei, and from the fact that they inherit chromatophores such as are found in the male but not in the female parent. With the general conclusion that the disturbance involves the relation of nucleus and cytoplasm, rather than the nucleus alone, my results are in complete accord.

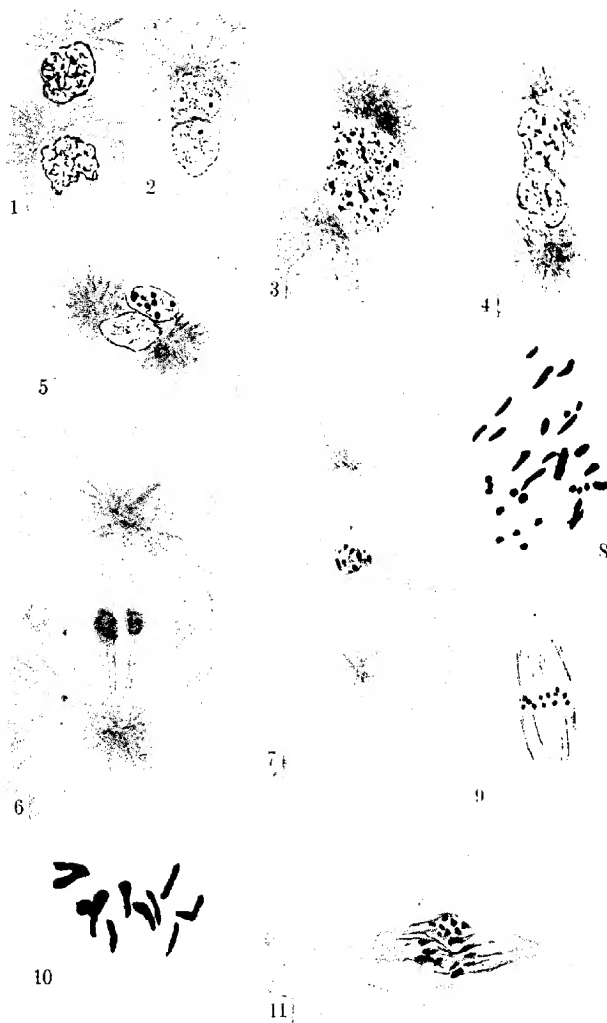
## EXPLANATION OF PLATES

The essential features of all figures were drawn with a camera lucida. Unless otherwise stated, the magnification is 1000 diameters.

### PLATE I

#### EXPLANATION OF FIGURES

- 1 F ♀ × C ♂; aster divided; germ-nuclei not yet closely apposed.
- 2 F ♀ × F ♂; germ-nuclei about to fuse; aster undivided.
- 3 F ♀ × F ♂; segmentation nucleus.
- 4 and 5 F ♀ × C ♂; germ-nuclei closely apposed but not fused.
- 6 F ♀ × C ♂; prophase of first cleavage; the nuclei have not fused.
- 7 F ♀ × F ♂; prophase of first cleavage; the nuclei have fused.
- 8 F ♀ × C ♂; early anaphase, first cleavage; paternal and maternal chromatin mingled (× 2500).
- 9 C ♀ × C ♂; metaphase, first cleavage.
- 10 F ♀ × F ♂; metaphase, first cleavage (× 2500).
- 11 F ♀ × C ♂; early anaphase, first cleavage; paternal chromosomes grouped on one side of the spindle.

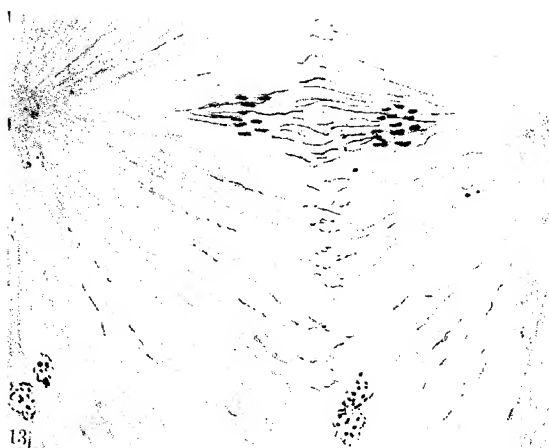
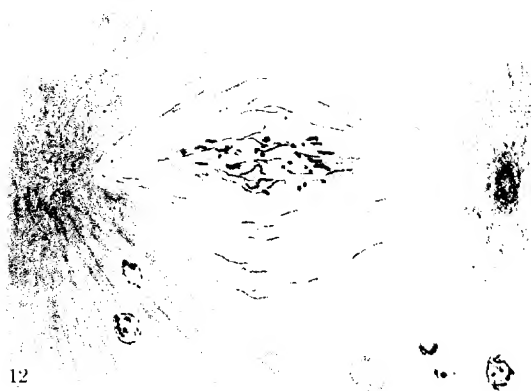




## PLATE 2

### EXPLANATION OF FIGURES

- 12 F ♀ × C ♂; anaphase, first cleavage.  
 13 F ♀ × F ♂; anaphase, first cleavage.



# PLATE 3

## EXPLANATION OF FIGURES

- 14 F ♀ × C ♂; anaphase, first cleavage (× 2500).
- 15 F ♀ × F ♂; anaphase, first cleavage (× 2500).
- 16 F ♀ × C ♂; anaphase, first cleavage (× 2500).
- 17 F ♀ × C ♂; anaphase, first cleavage.
- 18 F ♀ × F ♂; anaphase, first cleavage.
- 19 F ♀ × C ♂; anaphase, first cleavage.

BEHAVIOR OF CHROMATIN IN HYBRIDS  
MARGARET VOORIS

PLATE 5

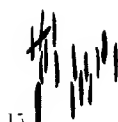
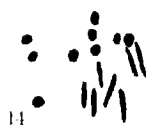
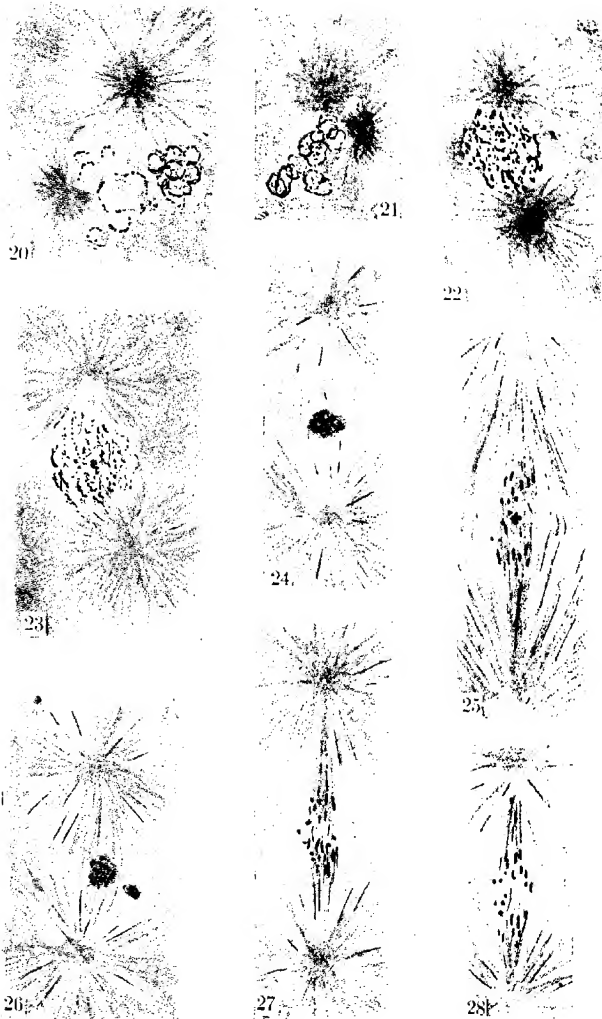


PLATE 4

EXPLANATION OF FIGURES

- 20 F ♀ × F ♂; telophase, first cleavage.
- 21 F ♀ × C ♂; telophase, first cleavage.
- 22 F ♀ × C ♂; nucleus, 2-cell stage.
- 23 F ♀ × F ♂; nucleus, 2-cell stage.
- 24 F ♀ × C ♂; prophase, second cleavage.
- 25 F ♀ × C ♂; anaphase, second cleavage.
- 26 F ♀ × C ♂; prophase, fourth cleavage.
- 27 F ♀ × C ♂; anaphase, fourth cleavage.
- 28 F ♀ × C ♂; anaphase, sixth cleavage.



## PLATE 5

### EXPLANATION OF FIGURES

- 29, 30, 31 and 32 F ♀  $\times$  C ♂; normally dividing cells from 12-hour stages.  
33 F ♀  $\times$  C ♂; large cells with irregular nuclei from 12-hour stage. A few normal cells are drawn for comparison ( $\times 350$ ).  
34 F ♀  $\times$  C ♂; abnormal cell from 12-hour stage.  
35 Fundulus sperm ( $\times 2500$ ).  
36 Ctenolabrus sperm ( $\times 2500$ ).



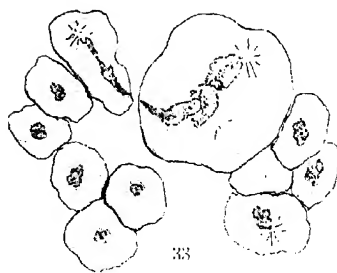
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# STUDIES OF FERTILIZATION

## VI. THE MECHANISM OF FERTILIZATION IN ARBACIA

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ONE FIGURE

### CONTENTS

I. Introduction.....	524
II. The spermophile side-chain.....	525
1. A quantitative method of studying the production of fertilizin by ova.....	525
2. The quantity of fertilizin produced by ova of Arbacia.....	528
a. By a single washing.....	528
b. Comparison of ripe and unripe eggs.....	530
c. Fertilizin produced in a series of washings: rôle of the jelly.....	532
3. The binding of fertilizin by sperm.....	535
4. Separateness of heteroagglutinating substances in egg-secretions.....	541
5. Some special properties of fertilizin.....	543
6. Anti-fertilizin.....	544
7. The necessity of fertilizin for fertilization.....	549
a. Fertilization of washed eggs.....	549
b. Cessation of fertilizin production after fertilization.....	553
c. Cessation of fertilizin production after formation of membranes by butyric acid.....	557
8. Summary of Part II.....	561
III. The ovophile side-chain.....	562
Introduction.....	562
1. The inhibiting effect of blood of the sea-urchin upon fertilization of eggs of the same species.....	563
2. Mode of action of the inhibitor.....	570
3. Summary of Part III.....	577
IV. General discussion.....	577
1. The mechanism of fertilization.....	578
2. The two phases of fertilization.....	582
3. The lysin theory of fertilization.....	584
4. The theory of parthenogenesis.....	587
V. Literature cited.....	589

## I. INTRODUCTION

The fifth study of this series on "The behavior of the spermatozoa of *Nereis* and *Arbacia* with special reference to egg extractives" forms the prelude to the present study. In that paper I dealt with the phenomena of activation and of aggregation (chemotaxis) of spermatozoa and with their agglutination by egg secretions of the same species. Regarding fertilization as involving chemical interaction of the ovum and the spermatozoon, I concluded from some of the modes of behavior of the spermatozoa, especially the agglutination phenomena, that "these small motile cells may prove better indicators of some of the reactions involved in fertilization than the slowly reacting egg" and that we might "confidently expect that study of the reactions of spermatozoa will break a new path into the field of fertilization."

The results described in the present paper have been obtained largely by the use of sperm-suspensions as indicators, but also by the study of an inhibitor of fertilization contained in the blood of certain individuals of the same species. These methods reveal a striking series of facts which appear to me to justify a somewhat new point of view in regard to some of the problems of fertilization. I have already briefly presented this point of view in a rather special form with an outline of the main facts in a preliminary paper (Lillie '13).

The essential conclusion is that fertilization is a reaction between three bodies of which one is born by the sperm and one by the egg; the third body, which is secreted by the egg, reacts with both the others. The spermatozoon functions essentially as an activator of the third body which I propose to name 'fertilizin';<sup>1</sup> the latter when activated enters into certain reactions in the cortex of the egg which lead to membrane formation. In order to give a concrete working conception I have pictured the fertilizin as possessing two side chains active in fertilization, viz: one

<sup>1</sup> This is the same substance which I called the 'sperm isoagglutinin' in the preceding paper of this series, because it causes agglutination in sperm suspensions of the same species.

reacting with the sperm which I call the 'spermophile side-chain' or group and the other reacting with the egg which I call the 'ovophile side-chain' or group. The chemical group of the sperm which reacts with the fertilizin is named the sperm receptor and that of the egg the egg receptor.

We are thus furnished with a concrete conception which answers very well for purposes of description; it need not however, be taken too literally. The theoretical aspect of the results is discussed in section IV.

The present paper presents in some detail the evidences on which this general conception of fertilization rests; it deals exclusively with *Arbacia*; in a later paper I shall present some confirmatory evidence from the study of *Nereis*. We shall consider first the action of the spermophile side-chain of the fertilizin and the evidence that the latter is a necessary link in the fertilization process. Second, we shall consider the nature of the ovophile side-chain of the fertilizin, the means of inhibiting its action and of neutralizing such inhibition.

The terminology has been largely adopted from immunology, because it seemed best suited to express the facts. If it seems rather bizarre to the zoological reader I must ask him not to conceive prejudice for this reason against the facts themselves, which stand on their own feet quite apart from any terminology or theory. The terminology and the theory are significant only to the extent that they give a brief description of the facts, and serve as a working hypothesis.

## II. THE SPERMOPHILE SIDE-CHAIN

### I. A QUANTITATIVE METHOD OF STUDYING THE PRODUCTION OF FERTILIZIN BY OVA

The fundamental fact which forms our point of departure is that fertilizable eggs of *Arbacia* suspended in sea-water secrete a substance (fertilizin) for which only the spermatozoa of the same species constitute an efficient indicator. Similarly the only cells that produce the substance are the egg-cells of the same species; it is not found in the blood or in extracts of other tissues.

The presence of the substance is shown by the agglutination of sperm-suspensions of the same species. If a few drops of sea-water containing this substance in sufficient concentration be added to a 1 per cent sperm-suspension in a test-tube a flocculent condition becomes apparent to the naked eye in a few moments. The qualitative aspects of the reaction were described quite fully in the preceding paper of this series, and need not therefore be repeated here.

In my previous papers ('12 and '13) no attempt was made to establish a quantitative method of study. But in order to carry the subject farther it was necessary to determine how much of the sperm agglutinating substance was produced by the ova under various conditions. As noted in previous papers the reaction is reversible. With high concentrations of the agglutinating substance it may be several minutes before the agglutinated masses break up and reversal is complete; with low concentrations on the other hand the agglutinated masses are smaller and their disintegration is correspondingly more rapid. It was therefore possible to establish as a unit concentration of the agglutinating substance the greatest dilution at which an unmistakable reaction is given. Such a reaction lasts only a few seconds. It would be obviously very tedious to establish the *exact* point at which the reaction ceases in a long series of experiments, and in practice I prepared a series of dilutions each member of which was one-half of the preceding member, and the last one to give the reaction was regarded as the unit concentration. The method is as follows: Taking a given solution of the fertilizin a series of dilutions is made. If these begin with (a) 1/100 (i.e., 99 parts of sea-water plus 1 part of the solution) a half dilution of (a) is made, (b) (i.e., 1/200); a half dilution of (b) follows (c) 1/400, (d) 1/800, (e) 1/1600, (f) 1/3200, etc. If 1/1600 is negative, 1/800 may give a 4 to 6-second reaction; if 1/800 is negative 1/400 may give a 4 to 6-second reaction, etc; when the solution was known to be strong it was usual to begin with a 1/100 dilution; otherwise we might begin with a 1/10 dilution, followed by a 1/20, 1/40, etc., or 1/2 followed by 1/4, 1/8, 1/16, etc., the last member of a series to give a positive reaction was accepted as

of unit concentration. Thus any given solution could be rated as of 10, 100, or 6400, etc., agglutinating strength depending on the dilution test.

The most delicate method of determining the reaction is the raised cover-slip method described in a previous paper ('13). The sperm-suspension used as indicator should be about 1 per cent (i.e., one drop dry sperm to about 99 drops sea-water) and should be not more than 10 minutes old. A few drops of this are mounted on a glass slide beneath a long cover-slip supported by glass rods about 1 to 1.5 mm. in diameter. The slide is placed on the stage of a microscope in focus under a magnification of about 40 diameters and a drop of the solution to be tested is blown into the sperm suspension with a capillary pipette attached to a flexible rubber tube. The agglutination reaction begins within less than a second if the solution be of considerable agglutinating power, or within 2 or 3 seconds if the solution is diluted nearly to the unit reaction. In the latter case it lasts only 4 to 6 seconds. The masses formed are relatively small in the latter case, and larger and denser proportionately with the higher concentrations.

The reaction is accompanied by a strong stimulation of the spermatozoa, and the reversal of the reaction by slackening of movement, which is more or less proportional to the strength of the solution, so that after agglutination with a strong solution the spermatozoa are practically paralyzed for a time. For this reason the agglutination masses formed by a strong solution do not break up readily and it is difficult to fix the exact cessation of the reaction; but with concentrations up to about 20 units the beginning and end of the reaction are both clearly marked. For a more detailed account of the reaction with higher concentrations, see Study V (Lillie '13, p. 550).

As an important detail of technique, I would say that for the determination of the more delicate reactions it is desirable to have the sperm suspensions free from blood corpuscles or other foreign particles. I therefore proceed as follows: the sea-urchin is dried slightly with a towel and is opened by a circular cut on the oral side of the equator. If it be a ripe male the thick sperm will begin to exude from the genital pores. The animal is then

laid aboral side down in a Syracuse watch crystal and the sperm allowed to collect in the crystal. In this way, as much as 2 cc. of thick perfectly clean sperm may be obtained from a large ripe male. If a few drops of sea-water have dripped off with the sperm it may be removed with a pipette, but if the sperm is abundant it is so dense that the fluid lies on it without intermingling. Such a mass of sperm will keep perfectly fresh for several hours, and suspensions may be made from it as needed for the tests. But the suspension used in testing must be freshly prepared if delicate reactions are desired.

The fertilizin is actively secreted by the eggs in sea-water as noted beyond; the concentration in the fluid of a given suspension of eggs in sea-water is a function of the quantity of eggs in the suspension. It will be convenient, therefore, in referring to any given agglutination solution, to have a method of expressing the relative quantity of eggs in proportion to the sea-water. The most convenient way of expressing this relation is to express the bulk of eggs as a percentage of the entire suspension. Thus 1 cc. of eggs added to 9 cc. of sea-water will be denominated a 10 per cent egg suspension, etc. This form of expression was not adopted until after the experiments were done; the figures are, therefore, not always even numbers, and are not intended in any case to be absolutely exact, but this is a matter of little importance as the density of a given egg suspension is only one factor in the concentration of the fertilizin in it; the condition of the eggs, whether entirely ripe or immature, fresh or stale, with or without jelly, is more important.

## 2. THE QUANTITY OF FERTILIZIN PRODUCED BY OVA OF ARBACIA

### *a. By a single washing*

July 6, 1913: For this experiment I used the eggs of a single female shed in a watch crystal in the same manner as described above for the collection of clean sperm. The eggs were thus free from blood or fragments of the ovaries and contained only a trace of the sea-water. Graded quantities of eggs were put in a series of six test-tubes containing 5 cc. of sea-water each. The eggs were placed in the tubes at 9:45 A.M.; each tube was turned upside down six times and the eggs then allowed to

settle; there was no shaking or rough handling of the eggs, so that they were absolutely uninjured. At 10:20 A.M., after the eggs had settled to the bottoms of the tubes, samples of the sea-water in the tubes 1 to 5 were removed and tested by a series of dilutions; at 11:45 A.M. a sample of 6 was taken and tested. The concentration of the sperm agglutinating substance in each is shown in table 1.

TABLE 1

TUBE	EGG SUSPENSIONS PER CENT	AGGLUTINATING STRENGTH OF SUPER- NATANT SEA-WATER	TESTS	
			Dilutions	Reactions
1	0.5	50	1/50	4-5 seconds
2	1.0	100	1/100	4-5 seconds
3	2.0	100 +	1/100	10 seconds
4	4.0	400	1/400	6 seconds
5	8.0	800	1/800	6 seconds
6	16.6	1600	1/1600	7-8 seconds

It is interesting to note that the quantity of fertilizin produced is so nearly proportional to the quantity of eggs. (No. 3 would probably have tested to 1/200 with perfectly fresh sperm.) We might expect in general that the quantity of fertilizin produced would continue to be proportional to the quantity of eggs. This however, may not be the case, as there are indications that in the presence of a certain concentration in the sea-water more is not secreted by the eggs.

It would appear that after the eggs had settled in the tubes into a dense layer on the bottom they ceased to produce more agglutinating substance, because the concentration in tube 6 in which the sea-water had stood over the eggs much longer than in tubes 1 to 5 was proportional to the quantity of eggs and not to the time.

Higher concentrations of the fertilizin were often obtained: August 9: A 25 per cent egg suspension stood for 12 minutes. The supernatant fluid then gave the following sperm-agglutination tests: diluted to 1/6400, 7-second reaction; 1/3200, 15-second reaction. August 18: egg suspension (density not given) was shaken. After settling of the eggs the supernatant fluid



gave the following sperm-agglutination tests: diluted to 1/12800, 5-second reaction; 1/6400, 10 to 12-second reaction.

Enough has been said to indicate the large amounts of this substance produced by the eggs in sea-water. No attempt has been made to produce the highest attainable concentration. The standardization of solutions for experiments was a daily procedure and in the experiments themselves hundreds of tests were made.

Hitherto, I think, the unfertilized egg has been generally regarded as a very inert cell, almost in a state of suspended animation. But it is necessary to modify such a point of view very greatly. The egg of *Arbacia*, at least, is a very actively secreting cell. Although the method which I have used enables us to calculate only the relative concentration of the agglutinating substance produced, yet the quantity must be very considerable when we consider that in tube 1, of the preceding experiment the egg suspension was only 0.5 per cent and that the supernatant fluid was charged with 50 units of the agglutinating substance in 35 minutes.

*b. Comparison of ripe and unripe eggs*

The tests already given are from fresh, ripe eggs. Ovaries containing a high proportion of ovocytes produce less fertilizin proportionately; but such ovaries could be obtained only at the beginning of the season so that only a few tests were made. On June 16 I compared equal amounts of ripe ovaries with practically all eggs mature, and unripe ovaries with only 50 per cent of the eggs mature. One part finely cut up ovary was added in each case to 3 parts sea-water. The first tested to 1/612, the second to 1/64. In a second determination the same day, in which *a* had only 5 per cent unripe eggs (ovocytes with germinal vesicle) and *b* had 50 per cent, one part ovary to 2 parts sea-water in each case, the tests were *a* 1/1000, *b* 1/600. In the latter case the strength of the solutions was about proportional to the percentage of mature eggs. In the former case the unripe ovary did not give as large a production of the fertilizin as would be expected

from the relative proportions of ripe eggs. Presumably, the ovarian tissue was relatively more bulky in this case.

The ovocytes are surrounded with the same layer of jelly in *Arbacia* as the mature eggs; hence we must conclude that the jelly is not the source of the agglutinating substance to whatever extent it may become charged with it after maturation. An important point is involved in this conclusion discussed farther on.

I think we are justified in concluding that ovocytes do not secrete the agglutinating substance. The conditions in *Nereis* fortify this conclusion, inasmuch as the ovocytes with intact germinal vesicle do not secrete the fertilizin, but as soon as insemination takes place it is poured out abundantly and the germinal vesicle breaks down.

In this connection it is important to note that in some animals ovocytes with intact germinal vesicle do not respond to the spermatozoon, even if cut in two so that the protoplasm is laid bare. Wilson ('03) has shown this for the nemertean, *Cerebratulus*, and Delage ('01) for the eggs of *Strongylocentrotus* and *Asterias*. As I show farther on that the fertilizin is necessary for fertilization, we may find in these facts evidence that the fertilizin is not only not secreted but is not even preformed in ovocytes with intact germinal vesicle, at any rate not in the forms mentioned. Its absence would furnish sufficient explanation of the failure of such egg-fragments to fertilize, and the consequent impossibility of producing merogony until the germinal vesicle breaks down.

Delage ('01) also shows that, as soon as the germinal vesicle begins to fade, in *Asterias*, and the nuclear sap diffuses into the cytoplasm, the egg becomes fertilizable and merogony can be induced. According to my interpretation the formation of fertilizin must begin at this time. At any rate the non-fertilizable condition of the cytoplasm before the germinal vesicle begins to fade is associated with the absence of fertilizin.

It is not necessary to assume that the fertilizin is contained in the nuclear sap; on the contrary, the long-continued production of this substance, as described in the next section, runs counter to this idea. It is more probable that it exists in a state comparable to zymogen ready to be converted into active form, and

that the escape of nuclear sap furnishes the necessary conditions. In some eggs, therefore, we might anticipate that sufficient substance might exude from the germinal vesicle without rupture to bring about formation of active fertilizin. Theoretically this should occur in such eggs as those of *Nereis* in which fertilization occurs before rupture of the germinal vesicle.

*c. Fertilizin produced in a series of washings; rôle of the jelly*

The production of this substance by the egg, in sea-water must be regarded as an active process of secretion, and, as will be seen immediately, it goes on continuously, in the case of *Arbacia*, as long as the eggs remain alive.

The last fact was ascertained in several attempts to remove all the fertilizin from the eggs by repeated washings. In these experiments it was also ascertained that while the jelly layer surrounding the eggs is saturated with fertilizin, eggs deprived of jelly still continue to produce it in considerable quantities. The method of making such tests is to suspend a measured amount of eggs in a given amount of sea-water and allow them to settle; as much of the supernatant fluid is then poured off as is possible without losing any eggs and kept for testing, and an equivalent amount of fresh sea-water replaced; the eggs are mixed up thoroughly, and allowed to settle, and the process is continued. In one series running three days in which the quantity of eggs was originally 2 cc. and the total volume of sea-water and eggs in the tube 10 cc., 6 to 8 cc. being poured off at each settling, thirty-four changes were made, and the agglutinating strength of the supernatant fluid diminished from 100 at first to 20 at the end. The details are given in table 2: Under the heading 'washings' the denominator gives the quantity of eggs and sea-water left after pouring off the supernatant fluid, and the numerator the total quantity after addition of the fresh sea-water.

This long series is amazing in its revelation of the extraordinary energy of the eggs in producing the fertilizin. In this case the eggs were allowed to retain their jelly, which, however, slowly dissolved until it was nearly all gone in washing 24.

TABLE 2

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 14			
1 10/3.0 cc.	1/100—10-12 sec.	2.0 cc.	11:01 A.M.
2 10/2.5 cc.	1/100— 8 sec.		
3 10/2.5 cc.	1/50 —10-11 sec.	1.15 cc.	11:12 A.M.
4 10/2.0 cc.	1/50 — 10 sec.	1.1 cc.	11:22 A.M.
5 10/2.0 cc.	1/100— 6 sec.		11:32 A.M.
6 10/2.0 cc.	1/50 — 9 sec.	1.2 cc.	1:55 P.M.
7 10/1.7 cc.	1/100— 12 sec.	0.9 cc.	2:20 P.M.
8 10/1.7 cc.	1/100— 7 sec.		2:35 P.M.
9 10/1.7 cc.	1/100 — 7 sec.		2:45 P.M.
10 10/1.7 cc.	1/100—11-13 sec.	0.8 cc.	2:53 P.M.
11 10/2.0 cc.	1/100— 11 sec.	0.8 cc.	3:03 P.M.
12 10/1.7 cc.	1/100 — 8 sec.	0.8 cc.	3:13 P.M.
13 10/1.5 cc. <sup>1</sup>	1/100— 12 sec.		3:23 P.M.
14 Fluid not changed <sup>2</sup>	1/100— 11 sec.		4:12 P.M.
August 15			
14a 10/2.8 cc.	1/50 slow atypical reaction		8:18 A.M.
15 10/1.9 cc.	1/100— 12 sec.	0.7 cc.	9:20 A.M.
16 10/2.0 cc.	1/100— 13 sec.	0.7 cc.	9:55 A.M.
17 10/2.0 cc.	1/100— 7 sec.	0.65 cc.	10:22 A.M.
18 10/2.0 cc.	1/25 — 7 sec.		10:40 A.M.
	(Note sudden fall 17-18)		
19 10/2.0 cc.	1/50 — 5 sec.		11:04 A.M.
20 10/2.0 cc.	1/25 — 5 sec.	0.6 cc.	11:34 A.M.
21 10/2.0 cc.	1/20 — 9 sec.	0.5 cc.	2:15 P.M.
22 10/4.0 cc.	1/20 — 7 sec.		2:45 P.M.
23 10/ .0 cc.	1/20 — 7 sec.	0.55 cc.	3:45 P.M.
24 10/2.0 cc.	1/20 — 7 sec.		4:25 P.M.
No jelly left on the eggs, except a thin layer on a few			
August 16 Continued the above series to 33 washings from 9:08 A.M. to 4:05 P.M.			
25			
26			
27	1/20 — 7 sec.		
28			
29			
30 <sup>3</sup>			
31	1/20 — 5 sec.		
32	1/10 — 10 sec.		
33	1/20 — 18 sec.		

<sup>1</sup> The jelly is now dissolved until only a thin layer is left. Washings 3-14 were absolutely colorless; and the following ones remained so until 35.

<sup>2</sup> The eggs were precipitated by slight centrifuging in the first 13 washings, except 6; in the others they were allowed to settle. The gradual reduction in bulk of the eggs is due to loss of jelly, not to loss of eggs.

<sup>3</sup> A drop from No. 30 was fertilized with excess of sperm at 11:36 A.M.; 5 per cent only segmented, irregularly; no membranes formed.

TABLE 2—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 17			
34 12/1.5 cc.	1/20 — 5 sec.	0.325 cc.	9:15 A.M.
35	On stirring up, the eggs disintegrate, and the fluid becomes highly colored; the agglutinating substance is <i>absolutely neutralized</i> . Test 1/1 negative, but the fluid is highly chemotactic and activating.		

In order to determine the rôle of the jelly in charging the sea-water with fertilizin, I killed some eggs by heating them for 5 minutes to 60° C. (August 15, 1913,) and submitted them to a series of 22 washings in 3 days, by which time the jelly was nearly all dissolved away: the agglutinating power decreased from 800 at first to 1 at the end. The agglutinating substance could be detected as long as any jelly remained.

On the other hand, eggs may be entirely deprived of jelly by shaking, and such eggs will continue to produce fertilizin as long as eggs with jelly, though in lesser quantities at first. This matter is so fundamental that I include another long table giving a comparison of eggs deprived of jelly by shaking and those with jelly (table 3).

August 21, 1913: In this experiment ripe eggs of *Arbacia* were washed once and divided in three equal lots, *A*, *B*, and *C*, in 10 cc. sea-water in graduated cylinders. They were allowed to settle, and at 9:20 A.M. the bulk of eggs was 1.8 cc. in *A*, 2 cc. in *B*, and 2 cc. in *C*. Thus there were about 10 per cent fewer eggs in *A* than in *B* and *C*. At 9:23 *A* was transferred to a test-tube and given 6 vigorous shakes to get rid of the jelly. A sample was then examined in India ink and it was found that 90 per cent of the eggs were entirely freed from the jelly; in the remainder it was usually reduced to a thin layer. *A* was then returned to its cylinder in 10 cc. sea-water and allowed to settle. As the eggs settled in *A* it could be seen that the fluid was only slightly colored; so that it was certain that not many eggs were injured in the shaking.<sup>2</sup> At 9:40 A.M. the bulk of eggs was as follows: *A*, 0.6 cc.; *B*, 1.6 cc.; *C*, 1.6 cc. As care was taken to avoid loss of eggs in *A* the

<sup>2</sup> It should be noted that in such an experiment, it is extremely important not to shake too much, for if the eggs themselves are injured so that the color escapes, a complicating factor comes in considered beyond (see section 6, p. 544).

decrease in bulk is due to loss of jelly, in *B* and *C* simply to further settling. *C* was kept for control. *A* and *B* were submitted to repeated washings as in table 3, p. 536.

This experiment positively decides the question as to production of fertilizin. It continues to be produced by the unfertilized eggs without jelly as long as they live. The jelly is, naturally, saturated with fertilizin, and it seems probable that it may act to prevent too rapid exhaustion of the secreting mechanism of the egg. It is of course possible that all that the egg gives off is pre-formed, but it is more probable that loss of this substance by the egg acts as stimulus to regeneration of more.

Apparently the eggs do not secrete the fertilizin in such a series of washings after they have settled in a mass at the bottom of the tube; for it will be noticed that the concentration of the substance in the successive washings is not a factor of the time intervals. On the contrary, a long time interval between washings is often associated with a temporary decrease in concentration, as may be noted, for instance, in the interval of 15 hours between 14 and 14a in table 2. In this case 14 and 14a are actually the same body of supernatant fluid and the concentration is much decreased in the second test 15 hours after the first, as though the eggs were gradually destroying the fertilizin. This subject is fully treated in section 6 (anti-fertilizin).

### 3. THE BINDING OF FERTILIZIN BY SPERM

In my previous paper (Lillie '13) I showed that the spermatozoa fix the agglutinating substance, and assumed as a working hypothesis that the fixation was due to chemical union. The fact on which the statement was based was the disappearance of the agglutinating substance from a sperm suspension when not present in excess. No attempt was made, however, to make quantitative determinations, so the matter was taken up again in 1913 in a more exact manner. The amount of the agglutinating substance actually fixed by the sperm is surprisingly small when the great avidity of the sperm for it is taken into account.

TABLE 3

WASHINGS		TESTS	BULK OF EGGS	TIME OF CHANGE
1913				
August 21				
1	A 10/2.0		0.6 cc.	9:42 A.M.
	B 10/3.0		1.6 cc.	9:42 A.M.
2	A 10/2.0		0.6 cc.	10:18 A.M.
	B 10/2.4		1.4 cc.	10:18 A.M.
3	A 10/2.6		0.55 cc.	10:40 A.M.
	B 10/3.0		1.4 cc.	10:40 A.M.
4	A 10/2.4		0.55 cc.	11:04 A.M.
	B 10/2.7		1.3 cc.	11:04 A.M.
5	A 10/3.0		0.55 cc.	11:27 A.M.
	B 10/2.6		1.2 cc.	11:27 A.M.
6	A 10/2.0		0.55 cc.	11:47 A.M.
	B 10/2.7		1.2 cc.	11:47 A.M.
7	A 10/2.0		0.5 cc. plus	2:03 P.M.
	B 10/1.6		0.8 cc.	2:03 P.M.
8	A 10/2.5	1/10 neg.; 1/1—50 sec.		2:30 P.M.
	B 10/2.5	1/20—13 sec.		
9	A 10/2.0			3:15 P.M.
	B 10/2.0			3:15 P.M.
10	A 10/2.0			3:45 P.M.
	B 10/2.3			3:45 P.M.
11	A 10/2.0			4:06 P.M.
	B 10/2.4			4:06 P.M.
Fertilization 1				
12	A 10/2.0			4:40 P.M.
	B 10/2.5			4:40 P.M.
13	A 10/1.5			7:32 P.M.
	B 10/1.2			7:32 P.M.
August 22				
14	A 10.4/3.4		0.4 cc.	8:18 A.M.
	B 10/2.8		0.6 cc.	8:18 A.M.
15	A 10/1.4			8:38 A.M.
	B 10/2.0			8:38 A.M.
16	A 11/1.8	1/10 trace; 1/1—60 sec.		9:13 A.M.
	B 11/2.2	1/10—20 sec.		9:13 A.M.
Fertilization 2				
17	A 10/1.7			9:40 A.M.
	B 10/2.5			9:40 A.M.
18	A 10/1.0			10:05 A.M.
	B 10.5/2.4			10:05 A.M.
19	A 10/1.0			10:23 A.M.
	B 10/3.0			10:23 A.M.

TABLE 3--Continued

WASHINGS		TESTS	BULK OF EGGS	TIME OF CHANGE
1913				
August 22				
20	A 10/1.0	1/10 trace; 1/1 trace and strong chemotaxis		10:48 A.M.
	B 10/2.4	1/10-9 sec.		
Note: Some eggs going to pieces in A				
21	A 10/1.5			11:10 A.M.
	B 10/3.0			
22	A 10/1.0			11:30 A.M.
	B 10/2.2			
23	A 10/1.4			2:12 P.M.
	B 10/2.0			
24	A 10/1.4	1/1 trace and strong chemotaxis		2:33 A.M.
	B 10/1.8	1/1-25 sec., slight chemotaxis		
25	A 10/1.0			2:55 P.M.
	B 10/1.2			
26	A 11/1.0			4:03 P.M.
	B 11/2.0			
27	A 10/1.0			4:19 P.M.
	B 10/2.0			
Fertilization 3				
28	A 10/2.0			4:36 P.M.
	B 10/1.0			
29	A 10/1.0			7:45 P.M.
	B 11/2.0			
August 23				
30	A 10/1.0	1/1-20 sec., chemotaxis <sup>1</sup>		8:37 A.M.
	B 10/2.5	1/1-30 sec., no chemotaxis		
31	A 10/1.5	1/1-14 sec. strong chemotaxis		9:13 A.M.
	B 10/2.0	1/1-20 sec. strong chemotaxis		
Fertilization 4				
32	A 10/1.0			10:10 A.M.
	B 10/2.0			
33	A 10/1.0	1/1-11 sec. strong chemotaxis		11:30 A.M.
	B 10/1.6	1/1-11 sec. strong chemotaxis		

<sup>1</sup> The chemotaxis noted in this series is due to injured eggs which secrete anti-fertilizin and conceal the fertilizin. See discussion.



TABLE 3—Continued

WASHINGS	TES	BULK OF EGGS	TIME OF CHANGE
1913			
August 23			
34 A 10/0.5			2:36 P.M.
B 10/1.0			
35 A; fluid not changed	1/1—11 sec.		
B fluid not changed	1/1—10 sec.		
August 24			
36 A; fluid not changed	1/1—8 second reaction		9:35 A.M.
B fluid not changed	1/1—7 second reaction		
37 Eggs shaken and broken; fluid not changed.			9:42 A.M.
A 1/1—negative			
B 1/1—4 sec.; 2d test negative			

Thus on August 13, I standardized an agglutination solution in sea-water to about 128 agglutinating power and placed 1 cc. in a tube (A). To this was added 1 cc. of a 3.3 per cent sperm suspension; 1 cc. of the agglutinated mixture A was then added to tube (B) containing 1 cc. of the same sperm suspension; 1 cc. of B was then added to 1 cc. of the same sperm suspension in tube (C). Thus the 128 power agglutinating solution was diluted with 3.3 per cent sperm twice in A, four times in B, and eight times in C.

- (A) 1 cc. 128 agglutinating power solution plus 1 cc. 3.3 per cent sperm
- (B) 1 cc. A plus 1 cc. 3.3 per cent sperm
- (C) 1 cc. B plus 1 cc. 3.3 per cent sperm

The sperm was then centrifuged off in the three tubes and on test the supernatant fluid was negative in C, faintly positive in B and gave a 60 to 80-second reaction in A. On renewed test, B was found almost negative. The result is then that 1 cc. of a 128 power agglutinating solution may be fixed by 3 cc. of 3.3 per cent sperm.

• Other tests of the binding power of sperm gave results of the same order of magnitude. Thus on August 9, I used a 64 power agglutinating solution as follows:

- (A) 1 cc. 64 agglutinating solution plus 1 cc. (approx.) 3 per cent sperm
- (B) 1 cc. A plus 1 cc. 3 per cent sperm
- (C) 1 cc. B plus 1 cc. 3 per cent sperm
- (D) 1 cc. C plus 1 cc. 3 per cent sperm

In this case after centrifuging off the sperm, *B*, *C* and *D* were negative; *A* was faintly positive. That is, 1 cc. of approximately 3 per cent sperm fixed 1 cc. of a 64 power agglutinating solution. This shows a greater binding power than in the previous experiment, but easily within the limits of error arising from difficulty of standardizing the sperm suspension, owing to the unavoidable inclusion of some fluid in the sperm. Other tests gave comparable results. Reduced to common terms:

August 9: 1 cc. 3 per cent sperm fixes 1 cc. 64 power agglutinating solution.

August 13: 1 cc. 3.3 per cent sperm fixes 0.66 cc. 64 power agglutinating solution

The difference is considerable, but when one considers first the difficulty of obtaining equivalent sperm suspensions and second the 2 to 1 method of standardizing the agglutinating solutions, and third, possible variations in the delicacy of the sperm suspensions used as indicators, the results lie well within the limit of error and indicate that there is a definite quantitative relation involved.

The possibility remains that the disappearance of the agglutinating substance in sperm suspensions is a phenomenon of adsorption rather than of chemical union. Experiments planned for an adequate test of this possibility were crowded out by other problems.

If sperm suspensions are allowed to stand for some hours they reach a condition when they will agglutinate only with strong solutions. If the binding power is then tested it is found that they fix as much of the agglutinating substance as before. The fixing power of the sperm is thus entirely independent of its capacity for being agglutinated, which is of course to be expected. But it is important, nevertheless, to emphasize it; the capacity for being agglutinated depends upon the motility of the spermatozoa, the binding capacity depends merely upon the presence of

chemical side-chains (receptors) capable of uniting with the agglutinating substance. When such a union takes place the heads of the spermatozoa must be supposed to become adhesive; if they move about rapidly, there are more collisions and hence more agglutination. With stale spermatozoa, either the movements are too slow to produce much agglutination, or the receptors are cast off and lie free in the medium, so that their binding does not affect the physical properties of the spermatozoa themselves. The latter possibility should be investigated, but was not this summer owing to the multitude of other problems.

A little light may be thrown on this problem by the results of June 26: In that case 4 drops of a strong, but not definitely standardized, solution of the agglutinating substance was added to 4 cc. of a 3 per cent sperm suspension 3 hours old. The tube was then centrifuged strongly and most of the spermatozoa precipitated in a mass at the bottom; however the supernatant fluid remained opalescent. Tested immediately with a fresh sperm suspension the fluid gave a 17 to 25-second agglutinating reaction. The tube was allowed to stand five minutes longer and was then tested again; the precipitated sperm remained in a solid mass, but the supernatant fluid had become negative; it contained no more free agglutinating substance. The quantity of sperm in suspension seemed much too small to account for the neutralization, which I am inclined to attribute to free receptors. But no really adequate test was made.

As to the interpretation to be given to the reversal of agglutination of the sperm it may be supposed either, (1) that the adhesive condition on which the agglutination depends exists only during an early union of the agglutinating substance with the sperm receptors and is lost as the union becomes firmer; or (2) that the bound receptors are cast off by the sperm. In view of the nature of the mechanism of the fertilization process, discussed beyond, I am inclined to lean to the second hypothesis.

4. SEPARATENESS OF HETERO-AGGLUTINATING SUBSTANCES IN  
EGG-SECRETIONS

I stated in my previous paper that in strong agglutinating solutions from *Arbacia* eggs and ovaries there is present also a substance that agglutinates the sperm of *Nereis*, and I argued that this was probably distinct from the iso-agglutinating substance because (1) it is contained in the blood of *Arbacia* which is normally entirely free from the iso-agglutinating substance, and (2) it may disappear from agglutinating solutions on standing while the iso-agglutinating substance remains apparently intact.

Some farther experiments made this year demonstrate the fact that the substance in agglutinating solutions from *Arbacia* eggs, that attacks *Nereis* sperm is a separate substance. We may note in the first place that the effect of this substance on the *Nereis* sperm is distinctly toxic. A drop containing it injected into a *Nereis* sperm suspension beneath a raised cover slip causes the formation of a permanent coagulated ring at the margin of the drop. In this respect the action differs from the iso-agglutination which is without toxic effects.

In the second place, the quantity present in strong iso-agglutinating solutions of *Arbacia* is extraordinarily variable; thus on June 18, 1913, an agglutinating solution was made by addition of 3 parts sea-water to 2 parts cut-up ovaries and eggs of *Arbacia*. The test on *Nereis* sperm showed: (1) undiluted, formation of a solid ring-coagulum; (2) diluted one-half with sea-water, solid ring-coagulum; (3) diluted to one-fourth with sea-water, slight permanent coagulum; (4) diluted to one-eighth, small coagulated masses; (5) diluted to one-sixteenth, nearly neutral; (6) diluted to one-twenty-fifth, absolutely neutral. Acting on *Arbacia* sperm the same solution gave a 5-second reaction at 1:200 dilution. Other samples equally strong in the iso-agglutinating substance were nearly neutral for *Nereis* sperm undiluted.

In the third place it is possible to precipitate out all of the *Nereis*-active substance with *Nereis* sperm and leave the full complement of iso-agglutinating substance. Thus on June 19 an agglutinating solution was made by addition of 3 parts sea-

water to 2 parts ovaries and eggs of *Arbacia*; the fluid was filtered off. Undiluted it had a powerful effect on *Nereis* sperm but was negative at 1/16 dilution; 1 cc. was then placed in each of three tubes *A*, *B*, *C*. To *A* was added 2 drops of an aggregative *Nereis* sperm-suspension, to *B* 4 drops of the same, to *C* 8 drops of the same. Precipitates formed in all. On testing the supernatant fluids all were found to be negative to *Nereis* sperm. The hetero-agglutinating substance was completely fixed even in *A*. Tested for the iso-agglutinating substance, *A* was shown to be equal in power to the control. The tests gave:

- (A) 1/800—faintest reaction
- Control 1/800—faintest reaction
- (A) 1/400—12 second reaction
- Control—1/400—8 second reaction

Thus while all the *Nereis*-active substance was destroyed the iso-active substance remained in full amount.

This is of course conclusive; but I was also able to show that the sperm of *Ctenolabrus* (teleost) may precipitate the hetero-active substance leaving the iso-agglutinating substance intact. The hetero-active substance would therefore appear to be rather generally toxic to foreign sperm. It is present in greater quantity in the blood than in egg solutions as shown by definite experiments which need not be quoted in detail. It is rather probable, indeed, that its presence in the egg solutions is due to contamination by the blood. But I did not test this out fully, though it could readily be done by successive washings of the eggs.

A rather embarrassing fact that appeared in the course of the above tests is that *Nereis* sperm may destroy considerable quantities of the iso-agglutinating substance if present in excess of the amount required to fix the hetero-active substance. This came out first in the test of *C* (under June 19 above), which contained at least 4 times the amount of *Nereis* sperm needed to fix the hetero-active substance, for the supernatant fluid in this tube turned out to be several times less active in its iso-agglutinating properties than the untreated control. It looked as though the *Nereis* sperm would combine more actively with the iso-agglutinating substance than the *Arbacia* sperm itself.

There are, however, strong reasons against accepting this conclusion: (1) there is no visible effect of the iso-agglutinating substance on the *Nereis* sperm as should be the case if there were actual combination. (2) The action of the *Nereis* sperm is ferment-like, inasmuch as the iso-agglutinating substance continues to disappear progressively for a long period of time in the presence of excess of *Nereis* sperm, whereas this is not the case in the combination with *Arbacia* sperm. I shall therefore omit the details of the experiments under this head, being convinced that we are dealing here with a case of actual destruction of the iso-agglutinating substance and not with chemical combination.

#### 5. SOME SPECIAL PROPERTIES OF THE FERTILIZIN

It would be interesting to know something concerning the chemical nature of this substance which is secreted by the eggs in such considerable quantities. This subject was, however, left for another occasion, as the investigation of its biological properties offered more problems than could be investigated at the time. Apart from these biological properties, all that I am now able to say about it is of a negative nature.

(a) It is colorless: Thus in table 2 all the washings after the first two were colorless though the concentration of the substance was considerable; similarly in table 3. (b) It must possess very considerable molecular size for it can be completely removed from any solution by filtering through a Berkefeld filter. Whether the solution be fresh or not, or of whatever concentration makes no difference; the filtrate obtained through a Berkefeld filter was invariably devoid of sperm agglutinating properties. On the other hand, it readily passes specially hardened filter paper. (c) Correspondingly it is non-dialyzable, not passing through the walls of celloidin tubes even in 24 hours. (d) It is extremely heat resistant being destroyed only slowly at the boiling point (see Study V, 1913). (e) As stated in my preliminary paper, I am indebted to Dr. Otto Glaser for the determination that it does not give the usual protein tests, even in the most concentrated solutions obtainable (see Glaser, 1914a). It is

thus colorless, non-filterable, non-dialyzable, very thermo-stable and apparently non-protein in its character.

The stability of the substance is also evidenced in its lasting qualities. Filtrates from egg-suspensions were kept and tested after various intervals of time. Thus the filtrate from a 33 per cent egg-suspension of June 21, was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. Similarly, the filtrate from a 33 per cent egg-suspension of June 20 was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. On the other hand, an agglutinating solution of June 30, of 800 agglutinating power was practically negative on August 26. In this tube there was more evidence of bacterial action than in the other two. Again, a distilled water extract kept from September, 1912, was of 16 agglutinating power on July 7, 1913.

In Study V, I also noted that its production is confined entirely to the eggs. It is not contained in the blood, and could not be extracted from other tissues.

#### 6. ANTI-FERTILIZIN

We have noted the extreme avidity of the spermatozoa for the fertilizin produced by the eggs. Neither the blood nor tissues appears to contain any substance capable of uniting with the spermophile side-chain which causes agglutination of the spermatozoa, or of neutralizing its action. However, such a substance is contained within the egg itself, and its discovery is one of the most interesting points in the mechanism of fertilization, because it would appear from the facts considered in section 7 that it is the occupancy of the spermophile side-chain of the fertilizin by this substance, which I propose to call anti-fertilizin, that prevents polyspermy, or refertilization of eggs.

Its presence can be demonstrated in the test-tube by methods which extract the internal substance of the eggs. But as the jelly surrounding the eggs contains an immense excess of fertilizin, complete neutralization of sperm agglutinating power can be obtained only after the jelly is removed, and still better if the eggs have been repeatedly washed until their fertilizin production is greatly reduced.

To consider the latter method first: The matter was first brought clearly to my attention in the experiment listed in table 2. In the 34th washing the supernatant fluid tested to 1/20 dilution; it was poured off, leaving 1.5 cc. in the tube, and 10.5 cc. of sea-water was added. When the eggs were stirred up they at once disintegrated, liberating the red pigment so that the fluid which in previous washings had been colorless became bright red. When this fluid was tested with a fresh sperm suspension it was absolutely without agglutinating effect. The dilution of the 20 power fluid of the 34th washing was 8 times, so that even if the eggs had suddenly ceased the production of the agglutinating substance the fluid would have contained 2.5 agglutinating units and should have given at least a 10-second agglutination reaction; but it was absolutely negative. The agglutinating substance had therefore been neutralized by some substance escaping from the disintegrating eggs.

In the experiment listed in table 3, the repetition of this result was of course looked for. On the morning of August 24, after 35 washings on previous days, I noticed that pigment was beginning to diffuse from the eggs in tubes *A* and *B*, but it had not extended above the 1.5 cc. mark. The eggs were beginning to break down. Without disturbing them, I tested the colorless supernatant fluid and got undiluted an 8-second reaction in *A* and a 7-second reaction in *B*. Both tubes were then vigorously shaken until the eggs were disintegrated and the fluid colored with the escaping pigment. Tested in 4 minutes *A* was negative for agglutination, *B* gave a 4-second reaction. Tube *B* was again shaken and in 11 minutes more its agglutinating action had also disappeared.

There can, therefore, be no doubt that the egg contains in its interior a substance that unites with the spermophile group of the fertilizin.

In these experiments it was interesting to note that after the agglutinating action had disappeared, the solution was very strongly chemotactic, so that the drop injected into the sperm suspensions formed a ring of active, entirely unagglutinated spermatozoa. The chemotactic substance is certainly distinct



from the fertilizin. The same observation was made also on other occasions.

The presence of the anti-fertilizin in the eggs could also be demonstrated in extracts of fresh eggs. These were made (1) by extracting in distilled water, (2) by drying eggs and grinding them in sea-water, (3) by grinding fresh eggs with sand in a mortar. I had previously often noticed that such extracts gave decidedly atypical agglutination of the spermatozoa, as compared with the action of the secretion of the eggs into sea-water. Distilled water extracts were then examined to find the cause for such atypical behavior. After it became clear from the experiments quoted above that the eggs contained the anti-fertilizin, the method of making the distilled water extracts was modified by first shaking the eggs as free from jelly as possible, so as to avoid the great excess of fertilizin contained in the jelly. These experiments yielded the following results:

August 22, 1913: 10:35 A.M. washed eggs were shaken six times in a test tube to get rid of the jelly. The eggs were washed twice again and shaken six times more; 60 per cent lost jelly; let stand. 2:15 P.M. Poured off sea-water to 2 cc. and added fresh sea-water to 8 cc. and divided equally in two test tubes, *A* and *B*; allowed to settle. 2:27 P.M. Poured off sea-water from settled eggs to 1 cc. in both *A* and *B*. To *A* added 7 cc. sea-water. To *B* added 7 cc. distilled water. The eggs were allowed to stand until they were completely plasmolyzed in *B* and the supernatant fluid was bright red. Tests were then made within an hour, or less, of the supernatant fluid in *A* and *B*, for the fertilizin, with somewhat surprising results:

(A) sea-water secretions  
Dilution tests

1/160—negative
1/80 —negative
1/40 —negative
1/20 —6 sec. reaction
1/1 —90 sec. reaction

(B) distilled water extract  
Dilution tests

1/1280—faint trace
1/640 —7 sec. reaction
1/320 —12 sec. reaction
1/160 —12 sec. reaction
1/80 —12 sec. reaction
1/40 —10 sec. reaction
1/20 —14 sec. reaction
(strong chemotaxis)
1/1 —12 sec. reaction
(strong chemotaxis)

Thus if only extreme dilutions were compared the conclusion would be that *B* is 32 times stronger than *A*, whereas comparison of the undi-

luted fluid would lead to the conclusion that *A* was  $7\frac{1}{2}$  times stronger than *B*. The fact that the time of the reaction of the *B* extract is about the same at all concentrations is in most pronounced contrast to the sea-water secretions in a very large number of tests, which had shown that the duration of the reaction is about proportional to the concentration of the agglutinating substance. The inference from the fact would be that the extract (*B*) contained an inhibitor for the agglutinating reaction which is diluted equally with the agglutinating substance. This might be a substance entering into combination with the agglutinating substance, but slowly and loosely at first.

4:07 P.M. Tube *B* was shaken vigorously to complete the plasmolysis. Tube *A* was stirred up. Both were then left until the next morning.

August 23, 1913: Tests of agglutinating power on fresh sperm:

<i>Tube A</i> <i>Dilution tests</i>	<i>Tube B</i> <i>Dilution tests</i>
1/20—6 sec.	Negative at all dilutions 1/1280.
1/10—12 sec.	1/640, 1/320, 1/160, 1/80, 1/40, 1/20.
1/1—190 sec.	1/10, 1/1, but showing decided chemotaxis.

Thus the neutralization of the fertilizin had become complete in the intervening time.

The disappearance of the agglutinating substance is not due to any destructive effect of distilled water on it, for control experiments showed that this was not the case.

A similar experiment on August 20 gave even more clear cut results. In this case (1) the eggs were deprived of jelly by 6 shakes, and examination of the eggs in ink showed that jelly was almost completely removed. (2) The eggs were allowed to settle and the supernatant fluid poured off. (3) The eggs were then divided into two equal parts in test-tubes *A* and *B*. (4) To *A* added 9 cc. fresh sea-water, to *B* added 9 cc. distilled water. (5) Each was stirred up twice and stood 30 minutes.

Tested with fresh 1 per cent sperm suspension, *A* gave a 10 second reaction at 1/40 dilution. *B* was negative at 1/2 and gave a faint reaction only, undiluted. Thus as *B* must have contained as much fertilizin as *A*, its disappearance must have been due to escape of anti-fertilizin by plasmolysis, inasmuch as we know that distilled water does not destroy it.

It would follow from this experiment in which the jelly was practically entirely removed, that the eggs contained an amount of anti-fertilizin sufficient to neutralize all the fertilizin present in them.

A third experiment yielded results similar to the first:

August 23, 1913: Fine lot of fresh eggs; shaken 6 times, 2:15 P.M. in test tube. Examination in ink showed about half have entirely lost

jelly, and that it is much reduced in nearly all. About 2 per cent of the eggs broken.

2:33 P.M. washed: Divided in two equal lots in test-tubes *A* and *B*. 3:08 P.M.: After setting of eggs poured off sea-water down to 2 cc. in each tube. To *A* added 6 cc. sea-water. To *B* added 6 cc. distilled water. Stirred several times until *B* was thoroughly laked.

Tests with fresh 1 per cent sperm

A. 3.40 P.M.	B. 4.05 to 4.15 P.M.	B. 4.35 P.M.
	1/640— 6 sec. reaction	1/640— 6 sec. reaction
	1/320— 7 sec. reaction	1/320— 8 sec. reaction
	1/160— 10 sec. reaction	1/160—12 sec. reaction
1/80— 4 sec. reaction	1/80 — 36 sec. reaction	1/80 —25 sec. reaction (chemotaxis)
1/40— 8 sec. reaction	1/40 — 60 sec. reaction	1/40 —22 sec. reaction
1/20— 13 sec. reaction	1/20 — 110 sec. reaction	1/20 —25 sec. reaction
1/10— 17 sec. reaction (1)	1/10 — 22 sec. reaction	1/10 —50 sec. reaction
1/1 —130 sec. reaction (1)	1/1 — 75 sec. reaction	1/1 —90 (?) sec. reaction

The second test of *B* indicated some progress in the neutralization of the fertilizin (cf. 1/20, 1/40, 1/80). The *B* dilution series is extremely irregular. Reading back from 1/1 (first test) the big jump from 1/10 to 1/20 indicates a breaking of the fertilizin X anti-fertilizin combination at this dilution.

August 24: Test showed some fertilizin present in *B*. Having poor sperm for indicator, left until next day. Shook up *B* vigorously; stirred *A*.

August 25: *B* now negative; *A* strongly agglutinative.

The distilled water extracts thus show the presence of a substance which combines quickly with the spermophile group of the fertilizin. In the experiment where the jelly was all removed by the preliminary shaking, there was practically immediate neutralization of the fertilizin. In the first and third, where the fertilizin was present in excess owing to the presence of some jelly, the excess was only slowly combined. Experiments with extracts of dried eggs (August 26) and with destruction of eggs by shaking or grinding also showed the presence of anti-fertilizin.

## 7. THE NECESSITY OF FERTILIZIN FOR FERTILIZATION

It will be apparent from the preceding facts and considerations that the fertilizin answers some of the requirements of a binding link between ovum and spermatozoon. When we consider the extraordinary activity of the unfertilized egg in its secretion, and the equally extraordinary avidity of the spermatozoa for it, one cannot escape the conviction that it must be a link in the normal fertilization process. When, moreover, one finds that the egg contains a more centrally located substance that can occupy the same combining group as the sperm, one seems to have before one, a view of a mechanism for preventing polyspermy.

I adopted, then, the working hypothesis that this substance is necessary for fertilization and there followed immediately three corollaries, viz: (1) If it were possible to extract this substance from eggs, they should no longer be capable of fertilization; (2) fertilized eggs are incapable of uniting again with spermatozoa, hence, if the hypothesis is correct, they could no longer contain free fertilizin; (3) eggs in which membranes have been formed by methods of artificial parthenogenesis become incapable of fertilization; such eggs must also, therefore, be devoid of free fertilizin after they have reached the non-fertilizable condition if the hypothesis is correct. These consequences of the theory were actually found to be true.

*a. Fertilization of washed eggs*

The only way of which I could think of extracting the fertilizin from the eggs without injury, is the method of repeated washings. But we have seen (tables 2 and 3) that it is actually impossible to remove all the fertilizin from a mass of eggs by any number of washings, for the eggs continue to produce it until they go to pieces. However, the quantity produced in successive washings slowly diminishes. Therefore, we might expect on the basis of the theory, that there would be a gradual reduction of the percentages of eggs fertilized after many repeated washings. This actually occurs, as table 4 shows. It might also be anticipated that as

the quantity of fertilizin decreases, the developmental energy of the fertilized eggs might decrease, owing to relative incompleteness of fertilization. This also occurs. In table 3 (p. 536) it will be seen that fertilizations were made at the times of the 11th, 16th, 27th and 31st washings. In each case about three drops of both egg-suspensions A and B were taken, including several hundred eggs and fertilized with considerable excess of fresh sperm. It will be recalled that the eggs of lot A had been deprived of their jelly by shaking, whereas those of lot B retained the jelly. A lost its fertilizin relatively rapidly therefore, at first, but later the repeated washings caused removal of all the jelly in B also.

Table 4 shows the fertilization results, as regards the percentage of eggs segmenting (200 eggs were counted in each case). There is thus the anticipated decrease in percentage of fertilizations. Moreover, the time between insemination and the appearance of cleavage increases very much, and the eggs cease after a while to produce fertilization membranes and the cells tend to fall apart.

TABLE 4

DATE		NUMBER OF WASHINGS	
August 21	4:06 P.M. Fert. 1.....	11	A 76.0% segmented B 97.5% segmented
August 22	9:13 A.M. Fert. 2.....	16	A 76.0% segmented B 92.0% segmented
August 22	4:19 P.M. Fert. 3.....	27	A 83.5% segmented B 97.5% segmented
August 23	9:13 A.M. Fert. 4.....	31	A 30.0% segmented B 30.0% segmented
August 23	11:30 A.M. Fert. 5.....	33	A 25.0% segmented B 24.0% segmented

Concomitantly, with these effects of the series of washings, the developmental energy becomes greatly reduced. This was very obvious from the second fertilization. On August 24 (48 hours after fertilization) a large quantity of living material was contained in the second A fertilization, but not one had even approximately pluteus structure. The most common form was a

stereoblastula. In the second *B* fertilization there were a few abnormal prismatic plutei, while the majority were gastrulae; some stereoblastulae. The third fertilization resulted in extremely abnormal ciliated types. The fourth and fifth did not proceed beyond abnormal cleavage stages.

The eggs have evidently lost something which affects their power of fertilization. Table 3 shows the measure of loss of the sperm-agglutinating substance, and justifies the general conclusion that this is a factor in the result. The loss of other substances may also combine in the decrease of fertilizing power, but of this we know nothing definite. As a matter of fact, fertilizing power is gradually lost with decrease of fertilizin content of the egg.

A considerable number of eggs go to pieces during the washings. This was especially noticeable in the *A* series of the above experiment. Thus after the 19th washing my notes record that eggs were stuck to the tube to the 3.5 cc. mark, whereas in *B* where the eggs were protected by jelly, only a very few stuck and these did not extend above the 1 cc. mark. Such eggs liberate antifertilizin, and if this is present in sufficient amount it may conceal the fertilizin production of the intact eggs. This phenomenon appeared a number of times in my earlier experiments when eggs were shaken too strongly in the effort to get rid of the jelly, resulting in injury to a large proportion of eggs, which soon broke down and neutralized the fertilizin produced by intact eggs.

One other experiment with apparently much more decisive results may be given (Aug. 18, 1913). In this experiment the shaking lasted 45 seconds and over 99 per cent of the eggs were stripped of jelly. The eggs were then precipitated by the centrifuge using just enough force to ensure complete precipitation, and the supernatant fluid poured off. It was very highly colored, showing that many eggs had been broken. It tested to a dilution of 1/12800, giving a 5 second reaction. The eggs were then repeatedly washed about 10/1.5 each time. The time of change and tests of supernatant fluids are shown in table 5, p. 552.

3:45 p.m. The fluid in 15 now gives a 6 second reaction, showing that some eggs are still producing fertilizin, but that it is rapidly neutralized.

After the 8th washing these eggs had extremely little capacity for fertilization, although only 24 hours old: Fertilization 1. 9:08 a.m. August 19: A few drops of stirred up egg suspension put in 9 cc. seawater and 3 drops 1 per cent sperm added. This is an enormous excess

TABLE 5

WASH- ING	TIME	TEST
2	2:48 P.M.	1/6400
3	3:15 P.M.	1/800
4	3:47 P.M.	1/100 (bulk of eggs 0.2 cc.)
5	4:03 P.M.	1/20
6	5:25 P.M.	1/10
7	Aug. 19: eggs had stood in tubes over night	
	8:37 A.M.	1/10 negative. 1/1—13 seconds
8	9:10 A.M.	1/10—11 seconds
9	9:42 A.M.	1/10—10 seconds
10	10:00 A.M.	1/10—(faint)
11	10:20 A.M.	1/1 —11 sec. (strong chemotaxis)
12	10:47 A.M.	1/1 — 9 sec. (strong chemotaxis)
13	11:08 A.M.	1/1 — 5 sec.
14	11:53 A.M.	1/1 — 4 sec.
15	2:00 A.M.	1/1 — (negative)

of sperm. The eggs were rapidly literally covered with a single layer of adherent spermatozoa, but in spite of this only about 1 per cent divided (out of 200 eggs counted 3 were divided irregularly at 11:15). Some fresh eggs fertilized for control of sperm at same time showed practically all eggs divided at this time. Fertilization 2. 10:02 A.M. Same as 1, with control: 2:03 P.M. About 1 per cent divided; irregular, delayed; 80 per cent of control. Fertilization 3. 11:02. Same as 1, with control: 2:15 P.M. About 2 per cent divided; control 99 per cent divided.

Thus these eggs had almost entirely lost the capacity for fertilization with their loss of fertilizin, although they were only 24 hours in the sea-water. However, I regard the result given in the first experiment under this head as more typical for mere loss of fertilizin. In the experiment just quoted, the eggs had been injured to such an extent that they were liberating anti-fertilizin also, and I believe that this combined with the fertilizin in the cortex of the egg so as to block fertilization in this way. The extraordinarily rapid decrease of agglutinating power of successive washings (1, 12,800; 2, 6400; 3, 800; 4, 100; 5, 20; 6, 10; 7, 2 (?); 8, 10; 9, 10; 10, 10; 11, 1; 12, 1; 13, 1; 14, 1; 15, 0) is such as occurs only with injured eggs.

Other experiments give similar results. In one of these, the fertilizing power was reduced to about 1/8 in the third washing

after violent shaking which removed the jelly and in which the agglutinating power had been reduced from 400 to 10. It would appear from this that it makes no difference how rapidly we dispose of the fertilizin; the eggs are no longer capable of fertilization when it is gone.

*b. Cessation of fertilizin production after fertilization*

In a previous paper (Lillie '13) I noted that eggs of *Nereis* cease to produce sperm-agglutinating substance soon after fertilization, but that apparently this was not the case in *Arbacia*. I, however, ventured the suggestion that the apparent continued production of the sperm-agglutinating substance in *Arbacia* might be due to the presence of unfertilized eggs, of which there was a considerable percentage in the cultures. This may have had something to do with the results there described, but the factor that leads to an apparent continuation of fertilizin production in fertilized eggs is its presence in high concentration in the jelly, a condition that I, by no means, realized at first. When this factor was eliminated it was easy to demonstrate that fertilized eggs no longer produce the substance. The crucial experiments were as follows:

Experiment 1: August 23, 1913: A large quantity of unusually fine eggs were washed three times and fertilized at 2:30 P.M. with considerable excess of sperm. At 2:40 P.M. microscopical observation showed that nearly all had beautifully formed fertilization membranes. About 10 per cent of the eggs were without jelly (observation in ink). These usually had as fine membranes as the others.

The eggs were divided in two equal quantities in test tubes *A* and *B*. 2:50 P.M.: *B* was shaken 6 times to get rid of the jelly. Examination in ink showed that the jelly was removed completely from practically all the eggs. *A* (with jelly left on eggs) and *B* were then submitted to a series of washings with tests for fertilizin as shown in table 6, p. 554.

Thus in five washings the *B* fertilized eggs without jelly no longer gave the fertilizin test. This number of washings would be only barely sufficient to remove the agglutinating substance present before fertilization and the loose jelly. Two successive negative tests were given, showing that the production of the agglutinating substance has ceased absolutely. The *A* eggs were



TABLE 8

3:10 P.M.	A 1	Washed 8/2.0	
	B 1	Washed 8/1.0	
3:25 P.M.	A 2	Washed 8/1.5	
	B 2	Washed 8/1.0	
3:43 P.M.	A 3	Washed 8/2.5	
	B 3	Washed 8/1.0	Test 1/1—22 seconds
4:08 P.M.	A 4	Washed 8/2.0	
	B 4	Washed 8/1.0	Test 1/1—9 seconds
4:53 P.M.	A 5	Washed 8/1.5	Test 1/1 over 75 seconds
	B 5	Washed 8/1.0	Test 1/1—negative
4:58 P.M.	A 5		Second test 1/1—60 seconds
	B 5		Second test 1/1—negative

apparently still producing it vigorously, but that is only because they carried the fertilizin-saturated jelly with them. Ninety-eight per cent of the *B* eggs segmented perfectly.

The result is in accord with the assumption that all free fertilizin is fixed *at the moment of fertilization*, or membrane formation. But I know of no way of absolutely demonstrating that the fixation takes place thus rapidly. But if such fixation is the mechanism for prevention of polyspermy, as I believe, the fixation of all free fertilizin must take place in a few seconds at most.

A duplicate experiment was made on August 25. The methods of Experiment 1 were repeated exactly. The eggs were not, however, so good a lot and the membranes did not stand put so far from the eggs. The shaking of lot *B* removed the jelly from most, but not from all. In this experiment tube *B* came negative on the sixth and seventh washings; whereas tube *A* gave a 90-second reaction, undiluted, on the sixth washing. Over 98 per cent of the eggs segmented and the unsegmented eggs had spindles.

Both experiments were clear-cut; there can be no question that after fertilization the eggs cease to produce fertilizin. Two effects are involved; the disappearance of fertilizin, and failure to produce more. The first effect might be due to extrusion of all fertilizin from the eggs; but it seems improbable that this could take place with sufficient rapidity to prove effective as a mechanism for preventing polyspermy, and inasmuch as we know that the eggs contain anti-fertilizin capable of occupying the spermo-

phie group of the fertilizin, it is more reasonable to conclude that the disappearance of sperm-agglutinating power from the washings of fertilized eggs deprived of jelly is due to neutralization of the substance remaining in the egg. In this connection it is instructive to note that eggs commonly lose pigment on insemination; I always have found the anti-fertilizin accompanied by pigment; it is, therefore, natural to suppose that anti-fertilizin is carried to the surface of the egg on insemination, thus affording the opportunity for combination with the fertilizin and its neutralization.

The other possibility that the sperm combines with all the free fertilizin in the egg appears to me inadmissible, because (1) the surface of the egg is relatively so enormous, and (2) the amount of fertilizin which the sperm is capable of binding is very small relative to the amount produced by the eggs even at a single washing (cf. sec. 3, p. 538). The bulk of the sperm actually used for insemination is insignificant compared to the bulk of the eggs, and only a small proportion of the sperm used actually fertilizes so that the amount fixed by the sperm itself is excessively minute compared to the quantity contained in the egg.

We have seen (p. 529) that 1 cc. of eggs charges 5 cc. of seawater to 1600 units. We have also seen that such eggs contain or produce many times this quantity in successive washings; and that it takes 1 cc. of 3 per cent sperm to fix 1 cc. of 64 power agglutinating solution. Thus it would require  $1 \text{ cc.} \times 1600 / 64 \times 5$  or 125 cc. of 3 per cent sperm to neutralize the agglutinating substance produced by one washing of 1 cc. of eggs in 5 cc. of seawater. But a single drop of 3 per cent sperm would be much more than sufficient to fertilize the same amount of eggs. The disproportion is even greater than indicated in this rough calculation, which is, however, sufficient to show the absurdity of explaining neutralization of the agglutinating substance of the fertilized eggs by the sperm used in fertilization.

Theories of prevention of polyspermy, or of the non-fertilizable character of eggs already fertilized, proceed along two lines: (1) that the membrane formed on fertilization is impermeable to spermatozoa, (2) that the protoplasm of the fertilized eggs has

undergone some 'physiological' change which prevents union with the sperm. The first theory is an old one based on conditions in the sea-urchins; it is inapplicable to the ova of most animals, which do not form special membranes after fertilization; whatever value the fertilization membrane may have in special cases as guarding against polyspermy, it has been shown for some cases at least that the real cause of failure of refertilization is more deeply seated. Thus Wilson ('03) shows for *Cerebratulus* that after the germinal vesicle has faded, cytoplasmic fragments devoid of nucleus may be fertilized and undergo typical merogonic development; but enucleated fragments of fertilized eggs are incapable of refertilization: "Even when such fragments are placed in water containing sperm immediately after the section before they have assumed a spherical form, they fail to fertilize, though spermatozoa may be observed adhering to their periphery." Wilson rightly attributes the sterility of such fragments to a physiological change of the cytoplasm.

The theory of a physiological change of the cytoplasm following fertilization has been generally accepted, but there has been no idea of the nature of the change involved. Wilson directs attention to the similar sterility of egg fragments prior to fading of the germinal vesicle and suggests that the cytoplasmic change following fertilization is physiologically the opposite of that following the fading of the germinal vesicle. Now, as I have shown the probability that the agglutinating substance is not present in an active form in *Arbacia* prior to fading of the germinal vesicle, and that it becomes neutralized by union with anti-fertilizin following fertilization, it is clear that this mechanism answers the requirements of the problem. The fundamental mechanism for the prevention of polyspermy is the neutralization of the fertilizin by the anti-fertilizin present in the egg; i.e., the occupancy of the spermophile side-chain of the fertilizin by the anti-fertilizin.<sup>3</sup>

<sup>3</sup> Note: Delage ('01) determined for *Asterias glacialis* that fragments of the oocyte with intact germinal vesicle are sterile, so that merogony cannot be produced. As soon as the germinal vesicle fades fragments are fertilizable and merogonic development may follow. Wilson's observations are in accord with

The question may be raised why such neutralization of the fertilizin is delayed until the moment of fertilization? The answer to this difficulty is fairly clear. The fertilizin is located in the cortex of the egg, and the anti-fertilizin is more deeply situated; they therefore do not interact so long as the cell body as a whole is quiescent. But as soon as the cortical fertilizin becomes activated by union with the sperm it at once begins to attack certain substances in the egg, as demonstrated in the third part of this paper; this sets up diffusion evidenced by escape of pigment, and by cytoplasmic flowing, and the two substances are brought together and interact. While this explanation is partly hypothetical, the spatial separation of the fertilizin and anti-fertilizin and the quiescent character of the cell-body in the unfertilized egg are facts; so also are the movements of diffusion and the cytoplasmic currents set up on fertilization. However it is possible that some other factor is operative in the interaction of fertilizin and anti-fertilizin following fertilization, which has entirely escaped attention.

*c. The cessation of fertilizin production after formation of membranes by butyric acid*

Some methods of artificial parthenogenesis cause eggs to become non-fertilizable; others apparently do not. In the sea-urchins it would seem that those methods that cause membrane formation result in the non-fertilizable condition while those which do not, need not have such an effect. Thus the use of hypertonic sea-water causes a certain amount of parthenogenesis

this as noted. But Delage farther asserts that a second change occurs after the formation of the second polar globule so that non-nucleated fragments again become sterile, although entire eggs can still be fertilized. This result is unsupported so far as I know, and would be difficult to explain on the basis of the mechanism described in this paper except on the assumption that the original contribution of nuclear sap necessary for the production of fertilizin has become exhausted, and that presence of the nucleus had become necessary for continued production of fertilizin. Delage shows that during the fertilizable period of the cytoplasm when merogony can be effected parthenogenetic agents are especially effective, a result that agrees particularly well with my point of view.

in sea-urchin eggs without membrane formation but the development tends to be quite abnormal. Loeb has observed that membrane formation may be induced in such eggs after they have reached even the 8 or 16-celled stage by addition of sperm; they are still fertilizable, so far at least as the cortical changes are concerned. It is fair to assume in such a case that the lack of membrane formation is evidence of persistence of free fertilizin in the cortex, for the only cases in which I found this substance absent from mature eggs are eggs with membranes formed. This case, therefore, need offer no difficulty for the contention that the fertilizin is necessary for fertilization.

On the other hand, Loeb's method of treatment with fatty acids leads to membrane formation and such eggs are non-fertilizable, as Loeb states and I have myself observed. Loeb, however, goes on to assert that if such eggs are shaken so as to destroy the membrane and if sperm is then added, a new membrane is formed and the typical development of sperm-fertilized eggs follows. This would seem at first sight to run counter to my theory unless it could be shown that membrane-formation by butyric acid differed from membrane formation by sperm in not causing cessation of fertilizin production. However, as I shall show immediately, the production of fertilizin does cease after the formation of good membranes by butyric acid.

It is necessary, therefore, to examine Loeb's statement rather carefully. It is as follows:

Dr. Kupelwieser und ich stellten diesen Versuch an, indem wir die Membranbildung bei unbefruchteten Eiern mittels Buttersäure hervorriefen, diese Membran dann *unmittelbar nach Bildung derselben*, durch Schütteln der Eier zum Platzen brachten und *sofort* Samen zufügten. Die Eier bildeten eine neue *dem Zytoplasma dicht anliegende Membran* und fürchten sich mit einer der Samenbefruchtung entsprechenden Geschwindigkeit, und Zwar zunächst ausnahmslos in zwei Zellen. (Loeb '09, p. 210; italics mine).

It is implied that such superposition of fertilization on parthenogenesis would not take place unless the experiment was performed immediately after membrane formation. Presumably some fertilizin remained unbound for a short time and on account

of its relatively small amount the new membrane was formed close to the egg. The implied fact that the experiment failed after a short time supports my theory of the function of fertilizin, and the stated result is not at all inconsistent with my views. According to my point of view the possibility of superimposing fertilization on parthenogenesis would persist so long as a sufficient quantity of fertilizin remained unbound, i.e., so long as the reaction was incomplete.<sup>4</sup>

The non-fertilizable character of such eggs implies on my theory an absence of free fertilizin. This condition obtains, as the following experiments show:

Experiment August 26, 1913: A quantity of fresh Arbacia eggs were taken and some were set aside in a test tube as control (0.14 cc. when settled). The balance were concentrated in 3.4 cc. sea-water.

3:47 P.M. The latter were then added to 25 cc. butyric acid in sea-water made by adding 2.8 cc.  $\frac{5}{16}$  butyric acid to 50 cc. sea-water.

3:47 1/2 P.M. Half of the eggs with acid were poured into 1500 cc. sea-water in crystallization dish A and stirred up to stop the action of the acid.

3:48 P.M. The remainder were poured into 1500 cc. sea-water in crystallization dish B and stirred up. B was over-exposed and the eggs agglutinated heavily. In A there was no agglutination and nearly all of the eggs formed fine membranes. The jelly was absolutely all gone owing to the action of the butyric acid, so there was no need to shake them.

The eggs in A and B were then concentrated, and placed in graduated tubes similar to the control. When settled there was 0.3 cc. eggs in each, estimated to be at least 4 times as many as in the control tube when allowance is made for the bulk of the jelly in the control. All were then submitted to successive washings with tests as shown in table 7, p. 560.

Thus the A eggs, with membranes formed, came negative in four washings, at least as rapidly as fertilized eggs, whereas the unfertilized control with fewer eggs was still showing an agglutination strength of about 10 (control 4, 1/1 = 50 seconds). There

<sup>4</sup> The essential problem is whether fertilization is of the nature of an irreversible chemical reaction, as I claim, or is merely a physical surface effect. Although this question has been investigated a good deal, it is still far from being definitively settled. The students of artificial parthenogenesis take the latter point of view generally.

TABLE 7

	WASHINGS	TESTS FOR FERTILIZIN
4:13 P.M.		
Control 1	10/1.2	
4:30 P.M.	A 1 12/2.4	Test 1/10—11 seconds
	B 1 12/1.5	
Control 2	11/2.0	Test 1/1 —2 plus min.
4:42 P.M.	A 2 13/2.0	Test 1/1 —32 seconds
	B 2 13/1.5	Test 1/1 —45 seconds
4:50 P.M.		
Control 3	10/1.0	No test
4:55 P.M.	A 3 13/2.0	Test 1/1 — 7 seconds
	B 3 13/2.0	Test 1/1 —11 seconds
5:05 P.M.		
Control 4	10/1.0	Test 1/1 —50 seconds
5:08 P.M.	A 4 12/2.0	Test 1/1 negative
5:13 P.M.	B 4 12/1.5	Test 1/1 — 9 seconds
Later	A 5 about the same	Test 1/1 —negative
	B 5 as above	Test 1/1 — 8 seconds

is no doubt, therefore, that in eggs with membranes formed by butyric acid the agglutinating substance is fixed, as it is in fertilized eggs. Presumably, the same would hold true for other parthenogenetic methods which render the eggs incapable of fertilization.

The next day the washings were continued. On the sixth, washing A again showed a trace of the agglutinating substance, but came negative again on the remaining three washings. The reappearance is to be attributed to the few eggs without membranes which had been active all night, accumulating in this time sufficient agglutinating substance to show in the test though not producing sufficient to show in repeated washings. The B eggs came negative in the ninth washing, while the control eggs still gave a 40-second reaction undiluted.

The B eggs although exposed longer to the butyric acid did not form membranes, and they continued to produce fertilizin longer than the A eggs but in much less quantity than the control eggs. It might therefore, be expected that they would retain capacity for fertilization longer. The fertilization capacities were not

worked out in this experiment; however, Herbst ('00) found in his experiments on superposition of fertilization on parthenogenesis that eggs overexposed to butyric acid could be fertilized, though incompletely.

Ninety-five per cent of the *A* eggs showed no evidence of cytotoxicity on the next day, beyond the membrane formation. They were intact and healthy looking. The remaining 5 per cent were fragmented, or segmented, into about 16 to 32 cells, within the membrane and appeared cytolyzed. In the *B* eggs the agglutination had disappeared, and there was about the same percentage of dividing eggs as in *A*, but in absence of a membrane the cells were breaking apart.

A similar experiment on August 25 gave the same result, viz., disappearance of the agglutinating substance after membrane formation by butyric acid.

Summarizing this section, then, we may say that eggs are rendered incapable of fertilization by methods that eliminate the sperm-agglutinating substance whether (a) the substance is lost, as in some eggs after repeated washings, or (b) fixed by previous fertilization, or (c) fixed by parthenogenetic methods. The conclusion is therefore justified that the agglutinating substance is necessary for fertilization.

#### 8. SUMMARY OF PART II: SPERMOPHILE GROUP OF THE FERTILIZIN

After considering a method for determining the quantities of fertilizin produced by ova of *Arbacia*, we demonstrated (1) that unfertilized eggs in sea-water produce this substance in extremely large quantities. The secretion begins presumably in the ovary with breaking of the germinal vesicle. Although the jelly is saturated with it and liberates it by solution in the sea-water, eggs without jelly continue to secrete it for three days, at least, during repeated washings, or as long as they remain alive. (2) The spermatozoa of *Arbacia* possess extreme avidity for the fertilizin and bind it in definite amounts. In the case of fresh active sperm the binding is evidenced by reversible agglutination of the spermatozoa. But a stale sperm suspension may also bind it, although



the agglutination does not take place, except in high concentrations. (3) The fertilizin of *Arbacia* eggs does not combine with *Nereis* sperm. (4) A substance, which I have named anti-fertilizin, may be extracted from *Arbacia* eggs, by breaking them up or by extracting them in distilled water, which has the property of combining with the spermophile group of the fertilizin. It is present in sufficient amount to neutralize all the fertilizin contained in the eggs, excluding the jelly. (5) When the fertilizin is extracted from the eggs by repeated washings, they are no longer capable of fertilization. (6) Fertilized eggs produce no more fertilizin; that present at the moment of fertilization is neutralized by anti-fertilizin. (7) Eggs with membranes formed by butyric acid become incapable of fertilization, and they contain no free fertilizin.

### III. THE OVOPHILE SIDE-CHAIN

#### INTRODUCTION

Having shown then, that the union of the agglutinating substance with the spermatozoon enters in some significant way into the process of fertilization, the problem was to ascertain in what way. The simplest idea (viz., that the union is itself the fertilization process) was soon shown to be untenable, for the reason that the perivisceral fluid (blood) of the sea-urchin especially of ripe males and females, often contains a substance which absolutely inhibits fertilization in the presence of any quantity of sperm, but which has no inhibiting effect at all upon the sperm-agglutinating reaction. It does not enter into combination with the spermophile side-chain. In other words, the binding of the agglutinating substance by the sperm may be complete, but in the presence of an inhibitor contained in the blood none of the usual effects of insemination, no matter how heavy, follow.

The next suggestion was fairly obvious, viz., that the substance which we have been calling the agglutinating substance on account of its effect on the spermatozoa, in reality possesses two side-chains active in fertilization, viz., a spermophile and an ovophile side-chain; the binding of the sperm activates the ovo-

phile combining group of the fertilizin which then seizes upon egg receptors, and it is the latter union which results in membrane formation. If this were so, it is obvious that the spermatozoon is only secondarily a fertilizing agent, in the sense of initiating development, and that the egg is in reality self-fertilizing, an idea that agrees well with the facts of parthenogenesis and with the amazing multiplicity of means by which parthenogenesis may be effected. For the agents need only facilitate the union of the fertilizin and egg receptor.

The inhibiting action of the blood, from this point of view, is a deviation effect due to occupancy of the ovophile group of the fertilizin, either because the inhibitor in the blood is an antibody to the fertilizin or because it possesses the same combining group as the egg receptor. In such a case, the ovophile group of the fertilizin being already occupied by the inhibitor, fertilization could not take place.

This theory transfers the fertilizing power from some hypothetical substance contained in the spermatozoon to a definite substance contained in the egg itself, in relation to which the sperm acts merely as an activator. It does not necessarily predicate the precise mode of action of the fertilizing substance although the assumption is here made that chemical union with certain molecules in the egg, called egg receptors, is a necessary part of its action.

In the present section we shall proceed to examine the evidence for the existence of an ovophile as well as a spermophile combining group of the fertilizin.

#### 1. THE INHIBITING EFFECT OF BLOOD OF THE SEA-URCHIN UPON FERTILIZATION OF EGGS OF THE SAME SPECIES

It is a common practice of embryologists to wash the eggs of marine invertebrates, which they propose to fertilize, twice or several times with sea-water, because it is a matter of experience that this procedure very greatly increases the proportion of eggs that fertilize. However, I know of no study of this really very striking phenomenon. The eggs are usually obtained for fertili-

zation by opening the animal and removing the ovaries so that the eggs are contaminated by blood or tissue secretions, and it is evident that the latter in some way inhibit the fertilization reaction, though they could hardly be supposed to have any direct injurious effect upon either sex element.

The necessity of washing the eggs carefully before fertilization, had been forced upon my attention by the poor results of certain experiments; and after some of the results recorded in the first part of the paper had been obtained, the phenomenon appeared to possess great significance, because the failure to fertilize in the case of unwashed eggs was clearly due to the presence of blood of the species. Some element of the blood must block the mechanism of fertilization, and it was clear that the analysis of the phenomenon must aid in understanding the mechanism.

The first experiments were undertaken to ascertain the extent of the inhibiting action of the blood of the sea-urchin. The nature of the effect may be seen from the protocol of the following experiment:

July 7, 1913: The blood of several males and females was obtained by cutting open the shell near the oral membrane and pouring out the perivisceral fluid (blood) in a finger-bowl. After the usual loose clot was formed the plasma was filtered through a soft filter-paper. The eggs used in the experiment came from one female and were washed in sea-water. The experiment was then set up as follows:

- 1 Quantity of eggs x plus 25 cc. sea-water plus 4 drops 1 per cent sperm
- 2 Quantity of eggs x plus 12.5 cc. filtered blood plus 12.5 cc. sea-water plus 4 drops 1 per cent sperm
- 3 Quantity of eggs  $2/5$  X plus 10 cc. filtered blood plus 4 drops 1 per cent sperm

The fertilizations were made about the same time and the result was that in 1 practically all divided, in 2 only a fraction of 1 per cent divided, in 3 none divided. Moreover in 2 and 3 no fertilization membranes were formed in the undivided eggs and the egg nucleus remained intact; no spindles were formed. And this in spite of the fact that the spermatozoa were very active, so abundant that they formed 'halos' around the eggs by penetrating the jelly, and many reached the surfaces of the eggs.

This experiment was followed by a considerable number carried out in more detail. Four of these experiments are tabulated in table 8. The left-hand column gives the percentage of blood in

the sea-water in which the fertilizations were made. The other vertical columns give the percentages of eggs segmented for each concentration of blood on the same horizontal level. The last reading in each column gives the control, i.e., fertilizations made in sea-water alone with the same amounts of the same eggs and sperm used in the blood solutions. The eggs in the blood solutions were washed in the same way as the controls prior to insemination.

TABLE 8

TABLE 8				
	JULY 8: MIXED BLOOD OF MALES AND FEMALES	JULY 15: BLOOD OF MALES ALONE		JULY 22: BLOOD OF MALES ALONE
Blood serum in sea-water	Segmented eggs			
		Exp. 1	Exp. 2	
1 per cent	75 per cent	90 per cent	90 per cent	89 per cent
3 per cent		25 per cent	60 per cent	21 per cent
5 per cent	10 per cent	10 per cent	30 per cent	0
7 per cent		1 per cent	30 per cent	6 per cent
9 per cent		25 per cent	45 per cent	19 per cent
10 per cent	about 0.2 per cent	50 per cent		
11 per cent			20 per cent	0
13 per cent			20 per cent	0.1 per cent
15 per cent			1.5 per cent	0
20 per cent	about 0.2 per cent			1.75 per cent
40 per cent	about 0.2 per cent			
100 per cent				0
Control: same eggs in sea- water	75 per cent	99 per cent	95 per cent	99 per cent

It will be noticed that in all these cases there is a marked reduction in the percentage of fertilizations when 5 per cent blood is present in the sea-water, greater in some cases than in others, which leads to the conclusion that the inhibitor is present in higher concentrations in some samples of blood than in others. This relation is dealt with below. In Experiments 1 and 2 of July 15, and in the experiment of July 22, there will be noticed after the first fall with increasing blood-concentration a rise and second fall in the percentages of fertilizations as measured by cleavage of the eggs. This is unquestionably of some significance, but I have been unable to ascertain what its meaning may be. The same phenomenon occurred in later experiments.

While the experiments cited give effects only of mixed blood from the two sexes, and the blood of the males alone, other experiments, not so complete in all details, showed that females contain the inhibitor in their blood at least to the same degree as the males. If there is any sexual difference it is in favor of the females. The blood of the males was used in many tests alone because there was danger, in opening the females, of wounding the ovaries and so getting fertilizin into the blood, which as shown beyond, greatly modifies the results. There was of course the danger in using blood from males of getting spermatozoa from wounded testes, but as the eggs were in any case to be fertilized the result could hardly be affected by this; some control experiments showed that this was a rare occurrence in any event.

Individual variability in the amount of the inhibitor present in the blood being indicated in these experiments, a special study was made of this factor in order to test its extent, and if possible to correlate it with the condition of the animals. The individual tests were as follows (table 9): the columns give the percentage of eggs segmented after 2 to 3 hours. All the fertilizations of July 28 were made with a single sperm suspension and a single lot of eggs; similarly for each succeeding date. The various numbers therefore act as controls on one another. In all, a great excess of sperm was used. The counts were carefully made and checked by my assistant, Miss Brockett.

These tests of bloods of individuals bring out very extreme individual differences in the content of fertilization inhibitor in the blood, ranging in fact all the way from no inhibition to absolute inhibition of fertilization. But it is not easy to correlate the variations with the conditions of the individuals. The general hypothesis that I have been led to adopt, provisionally at least, is that the quantity of the inhibitor in any individual blood is related to the size and degree of maturity of the gonads. It would seem to be a very simple matter to determine whether this is or is not the case. However, in the first place, after the beginning of the season there are practically no animals with immature gonads to test, and in the second place, if the hypothesis were correct, it would be impossible to know in advance how long the

TABLE 9

MALES—20% BLOOD		FEMALES—20% BLOOD		FEMALES—100% BLOOD
July 28				
1	65 per cent	1	75 per cent	11 per cent
2	69 per cent	2	93 per cent	84 per cent
3	71 per cent	3	72 per cent	11 per cent
4	72 per cent	4	45 per cent	1 per cent
5	85 per cent	5	85 per cent	84 per cent
6	82 per cent	6	87 per cent	63 per cent
7	27 per cent	7	89 per cent	81 per cent
8	84 per cent	8	52 per cent	0
		9	80 per cent	24 per cent
		10	92 per cent	93 per cent
		11	6 per cent	2 per cent
		12		55 per cent
		13		0
		14		most
		15		over 90 per cent (rough estimate)
MALES—100% BLOOD		FEMALES—100% BLOOD		
July 29				
9	100 per cent	16		77 per cent
10	85 per cent	17		39 per cent
11	56 per cent	18		100 per cent
12	87 per cent	19		0
13	43 per cent	20		100 per cent
Control 97 % (fertilized in sea-water)				
July 30				
14	2 per cent	21		35 per cent
15	25 per cent	22		100 per cent
16	29 per cent	23		15 per cent
17	93 per cent	24		65 per cent
18	93 per cent	25		19 per cent
19	100 per cent	26		99 per cent
		27		100 per cent
Control 100 per cent (fertilized in sea-water)				
July 31				
20	96 per cent	28		0
21	2 per cent	29		1 per cent
22	74 per cent	30		0
		31		38 per cent
		32		1 per cent
		33		41 per cent
		34		41 per cent
Control 94.5% (fertilized in sea-water)				
		35		89 per cent
		36		34 per cent

inhibitor might remain in the blood after the gonads were emptied. Thus the mere fact that an individual with small gonads had much inhibitor in its blood would not prove that the gonads had little to do with the formation of the inhibitor, for there would be no way of telling whether the reduction of the gonads was recent or not.

The considerations that led to the adoption of the above hypothesis are (1) that the condition of the gonads is the most variable thing in the summer sea-urchins, which coincides well with the variability of the amount of the inhibitor; (2) in general, the fluctuation through the season of periods of maturity of the sea-urchins coincides very well with the fluctuations in the amount of the inhibitor. Thus the animals were in very fine breeding condition during the first three weeks of July when the first tests showing abundance of the inhibitor in the blood, were made. They were in very poor condition then for about 10 days up to about August 2, when the individual tests were made showing such surprising decrease of the inhibitor. On August 6 when the material became better again, the inhibiting effect of the blood was stronger once more. (3) Although it was difficult, as said, in the individual tests to correlate the amount of the inhibitor with the condition of the animals, yet especially in the case of the females, those with large gonads appeared to have a large amount of inhibitor present. Thus females 28, 29, 30 and 32 were all ripe females that shed eggs when inverted after opening, in a watch crystal, whereas females 31, 33, 34, 35 and 36 were all individuals with medium size ovaries that shed no eggs when similarly inverted. Female 8, on the other hand, had small ovaries, shed no eggs, and yet possessed much inhibitor in its blood.

The conditions that determine the amount of inhibitor in the blood are thus a matter for more careful investigation. It may be possible to regulate the quantity experimentally by injection of fertilizin or of entire eggs. This merely indicates a possible method of approaching the problem.

I spent a great deal of time in endeavoring to meet the possible objection that the inhibiting action of the blood might be due to

contamination with the poisonous secretions of the epidermis, especially of pedicellariae. It seemed at first that this objection might be valid, for the period of my tests coincided with the season of poor material when the blood was relatively free from inhibitor. During this period, instead of following my earlier practice of opening the animal rapidly and pouring out the blood, I very carefully cut within the leathery peristome, avoiding all possible contamination with epidermal secretions and removed the blood with pipettes. This blood showed as a matter of fact little inhibiting effect. However, with the return of animals in good breeding condition, it was easy to show that the inhibitor was contained in the blood itself; and not only this, but also that epidermal secretions so far from increasing the inhibiting action of the blood, actually decreased it, a matter to which we return beyond.

The next question was whether the inhibitor acted on the egg alone, on the sperm alone, or merely by intervening in the reaction between the two? In the experiments undertaken to answer this question it was possible to show that the inhibitor certainly does not act strongly on either the egg or the spermatozoa alone. It is possible to collect eggs or sperm in filtered blood known to have a strong inhibiting action, and, after considerable exposure, to restore by washing nearly full fertilizing power, at least, to both kinds of sexual elements.

July 8: The blood used in this experiment allowed only 1 per cent fertilization when diluted to 5 per cent with sea-water; it therefore contained a great deal of the inhibitor.

1. As regards the effect on eggs alone: 9:41 A.M. The eggs of one female were placed direct from the ovary in some of this filtered blood. Transfers of two drops of eggs to 10 cc. of sea-water plus one drop 1 per cent sperm were made as follows: a. 9:42, b. 9:50, c. 9:55, d. 10:03, e. 10:23. About 60 per cent of each lot segmented. The blood carried over would be about 0.625 per cent. Thus the inhibiting effect was removed to a very great extent by washing. 2. In the case of sperm collected in the same blood and used for fertilization at 1 minute, 12 minutes, 22 minutes, and 37 minutes, no indication of inhibition of fertilizing power as compared with an identical series of controls was observed.

July 17: After 5 minutes' exposure to male blood sperm fertilized 20 per cent of the eggs; control sperm 90 per cent. (sperm = 0.004 per cent).



July 18: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 40 per cent in 5 minutes and 25 per cent in 18 minutes; (b) fertilized 30 per cent in 5 minutes and 20 per cent in 18 minutes. Control sperm fertilized 70 per cent each time. (Sperm = 0.0026 per cent)

July 19: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 14 per cent in 10 minutes and 18 per cent in 30 minutes; (b) fertilized 23 per cent in 10 minutes and 20 per cent in 30 minutes. Control sperm fertilized 86 per cent in 10 minutes and 76 per cent in 30 minutes. (Sperm = 0.012 per cent).

July 21: Sperm suspensions in blood of males. In this case the sperm fertilized perfectly in two fertilizations. Sperm = 0.007 per cent in first and 0.0068 per cent in second. The blood contained a large amount of inhibitor as only 0.5 per cent segmented in it with 0.6 per cent sperm suspension.

August 1: A very detailed experiment showed that a specimen of blood very powerful in inhibitor had but little effect on the fertilizing power either of eggs or of sperm after washing.

● On the whole we must conclude that though exposure to blood containing inhibitor may decrease the fertilizing power of sperm or of eggs, its effect is secondary and can probably be entirely removed by sufficient washing or overcome by sufficient concentration of sperm.

## 2. MODE OF ACTION OF THE INHIBITOR

It was natural to suppose that the inhibitor acted by occupying the side-chain of the fertilizin with which the sperm unites normally. If this were the case it must be possible to neutralize the agglutinating action of the fertilizin by a sufficient quantity of blood containing the inhibitor. Therefore, if we took two equal amounts of an agglutinating solution and diluted the one with sea-water and the other with blood containing the inhibitor, the agglutinating action ought to disappear more rapidly in the latter than in the former. This, however, is not the case, as the following experiment will show.

July 11, 1913: A given agglutinating solution was tested in the usual way by diluting with sea-water and found to be of 400 agglutinating power. Part of it was then diluted to 1/100 with blood; on test with sea-water sperm suspension it gave two tests of 22 seconds each. Diluted to 1/400 with blood, it gave a 10 second reaction with a sea-water sperm suspension, and an 11 second reaction with a blood sperm suspension.

The same result was obtained in other experiments and I could find no evidence that blood decreased the delicacy of the agglutinating reaction.

If then the fertilizin is essential for fertilization, and if the inhibitor in the blood does not operate by preventing union of this substance with the sperm, it follows that another side-chain of the fertilizin capable of being occupied by the inhibitor is also operative in fertilization. There must be action of the fertilizin on the egg as well as on the sperm, and hence we may assume an ovophile as well as a spermophile side-chain of the fertilizin, and the inhibitor must act by occupying the former.

Now Robertson ('12) has stated that various proteins when added to the sea-water can be shown to inhibit membrane formation by sperm, though as a matter of fact his experiments demonstrate such a result only for ovomucoid in concentrations above 0.25 per cent. It might therefore be supposed that the inhibiting effect of blood of the same species was only a special case of protein inhibition. There are, however, considerations that show such a view to be untenable. (1) As already shown the degree of inhibition by undiluted blood ranges all the way from zero to 100 per cent; and we cannot suppose that the amount of protein in the blood of different individuals varies to the extent required to explain such an extraordinary difference. (2) In the second place the mode of neutralizing the inhibitor, as shown below, involves an enormous increase of colloid content of the blood, for it consists in saturating the blood with fertilizin and dissolved jelly; the blood may thus become actually syrupy in consistency and yet have no inhibiting effect on fertilization. (3) I have tested the effect of egg-albumen in the sea-water up to 0.15 per cent without finding any inhibition of fertilization. Of course in higher concentrations a certain degree of inhibition may occur. However the reasons given under 1 and 2 above, certainly exclude the idea that we are dealing with an effect common to colloids in general, which must be assumed to operate through changes in the membrane. It is necessary to assume some specific substance acting as inhibitor.

Fortunately the idea that the inhibitor operates by occupying an ovophile side-chain of the fertilizin is capable of a ready test, for then it should be possible to neutralize the inhibiting effect of the blood, which now becomes ex. hyp. a deviating effect, by treating the inhibiting blood with free fertilizin. This substance should bind all molecules of the inhibitor so that they could no longer exert any deviating effect, their bonds for fertilizin being already occupied. This experiment succeeds perfectly as a matter of fact.

Before citing the experiments in detail, I would like to dwell a little more on their significance. In the first place a negative result would go far to overthrow the entire theory, for then it would become necessary to assume, if the theory were to be retained, that the combination of fertilizin and inhibitor could take place in the egg but not in the test tube, an exceedingly improbable assumption. The positive result, actually obtained, is in accord with the theory, and it is exceedingly difficult to frame any other interpretation of such a result. The alternative point of view would be to postulate a neutralization of a hypothetical membrane effect of the inhibitor, a conception exceedingly difficult to support as we have seen, and now rendered doubly difficult through the neutralization of such effect by excess of a substance which by itself in high concentrations actually reduces the percentage of fertilizations. Such an alternative interpretation appears to me impossible.

#### *The experiments*

July 21, 1913: The following substances were prepared: (1) Fresh filtered blood of male sea-urchins. (2) Filtrate from 15 cc. sea-water plus 7.5 cc. ripe Arbacia eggs, i.e., an agglutinating solution in sea-water, which gave an 8 second reaction with fresh sperm at 1/1600 dilution. (3) Filtrate from 15 cc. of the same male blood as in 1, plus 7.5 cc. eggs; i.e., part of the blood (1) saturated with the egg secretions. It gave a 10 second agglutinating test at 1/1600 dilution.

Two drops of fresh sperm (50 per cent) were then stirred into 5 cc. of each of the above and into (4) 5 cc. sea-water for control. These sperm suspensions prepared at 10:50 A.M. were thus about 0.6 per cent. One drop of double washed eggs was then added to each sperm suspension 1-4 at 11:45.

TABLE 10

		SEGMENTED EGGS
(1)	0.6 per cent sperm suspension in blood.....	0.5 per cent
(2)	0.6 per cent sperm suspension in 1600 agglut. power sea-water.....	99.0 + per cent
(3)	0.6 per cent sperm suspension in 1600 agglut. power blood.....	100.0 per cent
(4)	0.6 per cent sperm suspension in sea-water (control).....	100.0 per cent

This experiment was unusually successful; the eggs and sperm were perfect, giving 100 per cent segmentations (4). The blood contained so much inhibitor that only 0.5 per cent of the eggs segmented (1). But its action was entirely neutralized by the addition of the agglutinating substance (3). Fertilization 2 shows that in the presence of such an excess of sperm, the agglutinating substance itself has but little inhibiting power.

In a similar experiment of July 19, 0.9 per cent sperm suspensions were made in solutions 1 to 6 below, and a drop of eggs added to each with results noted in table 11.

TABLE 11

FERTILIZATIONS MADE IN		PERCENTAGE OF SEGMENTED EGGS
(1)	Filtered blood of male sea-urchins.....	0
(2)	Filtered blood of female sea-urchins.....	0
(3)	800 power agglutinating solution in sea-water.....	93%
(4)	1600 power agglutinating solution in mixed blood of males and females.....	73%
(5)	Mixture of equal parts of 1 and 3.....	0
(6)	50% dilution of 1 with sea-water.....	0
(7)	Sea-water control.....	97%

1, 2 and 6 show the inhibiting power of the blood on fertilization even with such enormous excess of sperm; 4 shows neutralization of the inhibiting power by a large quantity of the agglutinating substance although the neutralization was apparently not complete (cf. control); 5 shows that the agglutinating substance contained in an equal part of 800 power sea-water solution was inadequate to neutralize the inhibitor in this sample of blood; a considerable part of the inhibitor must have remained free; it must be present therefore, in high concentration.

Experiments were then made to test the neutralization of the inhibiting power of the blood at various dilutions.

July 22: Used filtered male blood which permitted only 0.5 per cent fertilization. Part of this was saturated with agglutinating substance by addition of 50 per cent by bulk of eggs, and filtered. Fertilizations were then made in a series of sea-water dilutions of each, using a single lot of eggs and of sperm (table 12).

TABLE 12

A SERIES—BLOOD ALONE			B SERIES—BLOOD SATURATED WITH AGGLUTINATING SUBSTANCE ABOUT 6400 STRONG		
Fertilizations in 0.1% sperm suspensions in		Per cent segmented	Fertilizations in 0.1% sperm suspensions in		Per cent segmented
(1)	1% blood	89.0%	(1)	1%	99.0%
(2)	3%	21.0%	(2)	3%	89.5%
(3)	5%	0	(3)	5%	97.0%
(4)	7%	6.0%	(4)	7%	94.5%
(5)	9%	19.0%	(5)	9%	93.5%
(6)	11%	0	(6)	11%	94.0%
(7)	13%	0.1%	(7)	13%	87.5%
(8)	15%	0	(8)	15%	91.0%
(9)	20%	1.75%	(9)	20%	71.0%
(10)	100%	0	(10)	100%	3.0%
Control in sea-water 99%					

It will be seen that this sample of blood is very powerful in inhibitor (A series), and that the agglutinating substance protects from its action at every concentration. The decreases in percentage of segmented eggs in B9 and B10 are to be attributed probably to excess of the agglutinating substance, which was exceedingly concentrated, and which always has some diminishing effect on percentage of fertilizations when the sperm suspension is not too concentrated. In this experiment it will be noted that the agglutinating substance was 6400 strong as contrasted with 1600 on July 21, and the sperm suspension was six times more dilute.

July 25: (A) Used filtered blood of females; (B) part of A saturated with agglutinating substance by addition of eggs; tested 3200 strong. Fertilizations were made in 0.2 per cent sperm suspensions in the following dilutions of each, with per cent of segmented eggs as noted, (table 13):

TABLE 13

A SERIES			B SERIES		
	DILUTIONS	PERCENTAGE OF SEGMENTED EGGS		DILUTIONS	PERCENTAGE OF SEGMENTED EGGS
(1)	10%	17%	(1)	10%	73.5%
(2)	40%	5%	(2)	40%	58%
(3)	70%	0	(3)	70%	40%
(4)	100%	0	(4)	100%	6%
(5)	Control in sea-water 58.5%				

The same protective action of the agglutinating substance is shown, with some decrease at the higher concentrations due, presumably, to excess of the agglutinating substance. The fertilization B-1 actually is better than the control.

August 6: This experiment was made up of a series of combinations of blood A plus fertilizin-saturated blood B. Fertilizations made in 0.2 per cent sperm suspensions in each combination.

TABLE 14

(A) Filtered serum of males and females  
(B) 23 cc. A plus 11.5 cc. ripe eggs. Centrifuged after 10 min. and filtered;  
sperm-agglutination test,  $1/3200=9$  sec.

FERTILIZATIONS IN		PERCENTAGES OF FERTILIZATION: 400 OF EACH COUNTED, 200 BY MEN, 200 BY HELP
	5.0 cc. A + 0 B	
(1)	4.9 cc. A + 0.1 cc. B	0.5%
(2)	4.8 cc. A + 0.2 cc. B	0.5%
(3)	4.7 cc. A + 0.3 cc. B	1.75%
(4)	4.6 cc. A + 0.4 cc. B	8.5%
(5)	4.5 cc. A + 0.5 cc. B	25.0%
(6)	4.4 cc. A + 0.6 cc. B	32.75%
(7)	4.3 cc. A + 0.7 cc. B	31.75%
(8)	4.2 cc. A + 0.8 cc. B	72.0%
(9)	4.1 cc. A + 0.9 cc. B	62.25%
(10)	4.0 cc. A + 1.0 cc. B	85.0%
(11)	3.0 cc. A + 2.0 cc. B	82.75%
(12)	2.0 cc. A + 3.0 cc. B	80.5%
(13)	0 A + 5.0 cc. B	50.0%
(14)		30.25%
Control in sea-water		89.5%

The protective action of the agglutinating substance gradually rises up to number 10 and then falls off, owing, presumably, to excess of agglutinating substance.

I have given a considerable number of the experiments because the matter under consideration is of great significance. There can be no question that some substance derived from the eggs themselves protects against the inhibiting substance in the blood. Until some method is devised for obtaining the agglutinating substance pure, it is impossible to meet entirely the objection that it may be some substance other than the agglutinating substance that protects against the inhibitor. The agglutinating substance can, however, be demonstrated in high concentrations, as the protocols of the experiments show. Until some reason for a contrary assumption is shown, the neutralization of the inhibitor

must be attributed to this, which is indeed the only assumption consistent with the other known facts.

Now if the agglutinating substance protects from the inhibitor by occupying its combining group, it would be logical to expect to find in some tissue of the species another kind of molecule possessing an identical side-chain and hence equally capable of protecting against the inhibitor. Such a substance was found in the shell but not in the intestine. A sample experiment may be cited.

August 2, 1913: A large quantity of blood was collected from females, and the serum filtered. (A) One part set aside. (B) A second part was saturated with the agglutinating substance by addition of eggs; *B* tested to 1/3200; (C) To a third part was added a large quantity of the shell and peristomes of the sea-urchins used: *B* and *C* filtered again.

Fertilizations were then made in 0.2 per cent sperm suspensions in identical concentrations of each in sea-water as shown in table 15.

TABLE 15

	DILUTIONS OF SERA	PERCENTAGES OF EGGS SEGMENTED		
		A	B	C
(1)	1%	98.0%	98%	97%
(2)	10%	94.0%	99+	96%
(3)	50%	58.5%	96%	99%
(4)	100%	15.0%	94%	97%
Control fertilized in sea-water		99%		

The conclusion would seem to be that shell secretions (poison from pedicellariae?) protect against the inhibitor in the blood. The inhibitor was not very strong in this case, and test of *C* showed that it contained a small quantity of agglutinating substance owing, as sometimes happened, to injuries to the ovaries in collecting the blood, but there did not seem to be enough (7 second reaction undiluted) to influence the result.

The above result was obtained in an attempt to meet the possible objection that the inhibiting action of the blood might be due to the inclusion of poison from pedicellariae. It appears, on the contrary, that shell-secretions decrease the inhibiting action of the blood, an entirely unexpected result.

As the result met the anticipated objection and did not appear to lie in the main line of the research, the experiment was performed only three times in all, with essentially similar results.

To explain such a result one of two hypotheses is possible: (1) that shell secretions destroy the inhibitor; (2) that they unite with its inhibiting side-chain, and thus possess a combining group identical with the ovophile group of the fertilizin; though they do not possess the spermophile group. The reaction takes place rapidly; hence the second assumption is more likely.

### 3. SUMMARY OF PART III

In this section we have shown:

1. That the blood of male and female sea-urchins contains a varying amount of a substance which inhibits fertilization without exerting any injurious effect upon either the sperm or the ova. This substance is probably more abundant in the blood of mature individuals than in others.

2. The inhibitor does not act by blocking the combination of the sperm receptors with the fertilizin, for it has no inhibiting effect upon the agglutinating action of the fertilizin.

3. It acts by occupying the ovophile group of the fertilizin, thus preventing action of the latter upon the egg by union with egg receptors.

4. This conclusion is demonstrated by the fact that the inhibitor may be entirely neutralized by a sufficient quantity of the agglutinating substance.

### IV. GENERAL DISCUSSION

The essential conception that results from the experiments is that the formation of the fertilization membrane is due to activation of an ovogenous substance, which I have named fertilizin to express this idea. Following insemination any excess of the fertilizin is bound, or neutralized, by another ovogenous substance, which I have named anti-fertilizin, and polyspermy is thereby prevented. The experiments demonstrate that reactions in fertilization occur as described in sections II and III, but the



intimate nature of the reactions themselves is unknown. To represent them in terms of the Ehrlich hypothesis as definite lock-and-key chemical combinations is of course to go beyond the facts. But it is necessary in some way to gain definiteness of formulation not only for purposes of description, but also to obtain a definite working hypothesis. The method of formulation which I have adopted has both of these advantages. Indeed some of the experiments could hardly have been suggested by any other hypothesis.

# 1. THE MECHANISM OF FERTILIZATION

In terms of such an hypothesis the mechanism of fertilization may be summed up in a diagram (fig. 1, sector 1). The cortex of the egg contains the fertilizin (see explanation of symbols) of which three molecules are represented each with a spermophile

Fig. 1 In successive sectors of the egg there are represented the mechanism of fertilization and the blocks to the mechanism, as follows:

Sector 1 The arrangement of substances in the unfertilized egg and in the spermatozoon that are active in fertilization. See explanation of symbols.

Sector 2 The mechanism of normal fertilization. The sperm receptor unites with the spermophile group of the fertilizin and the egg-receptors with the ovophile group of the fertilizin owing to activation of the latter by the sperm (a). Molecules of the anti-fertilizin combine with the spermophile group of the adjacent fertilizin (b and c) and thus block the way for supernumerary spermatozoa. This is the postulated mechanism for prevention of polyspermy. At the same time molecules b and c of the fertilizin have also united with the egg-receptors.

Sector 3 Inhibition of fertilization by loss of the active body, fertilizin.

Sector 4 Theory of antagonistic action of spermatozoa of different phyla. The sperm receptors are occupied by combining groups cast off by the antagonistic spermatozoa.

Sector 5 Fertilization is blocked by occupancy of the egg-receptors. Purely hypothetical.

Sector 6 Theory of inhibitory action of blood of the same species. The ovophile group of the fertilizin is occupied by molecules in the blood (inhibitor) possessing the same combining group as the egg-receptors. Molecules of the blood inhibitor also shown in the medium.

The fertilizin is represented in the diagram as occurring only in the cortex of the egg. But it also occurs in high concentration in the jelly surrounding the egg. The spermatozoon must thus normally arrive at the egg-membrane loaded with combined fertilizin. This fact, however, makes no essential difference in the theory, and its representation would complicate the diagram.

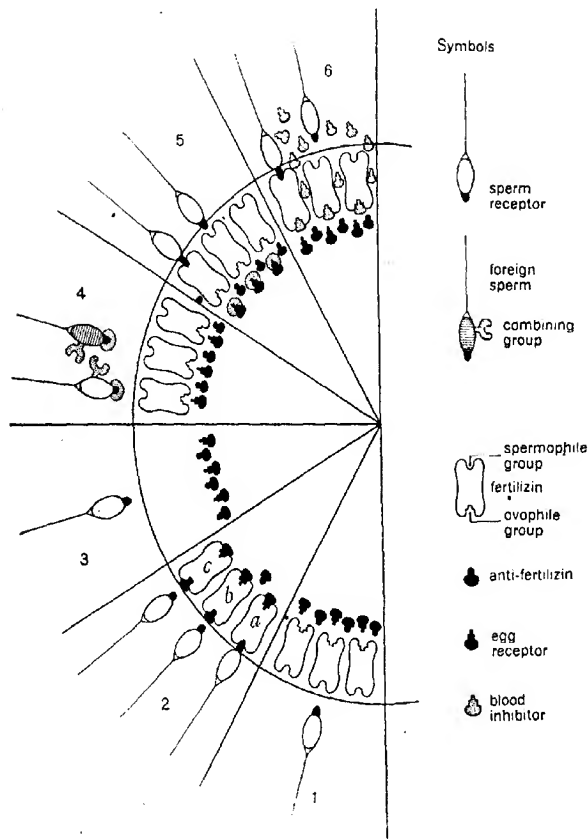


Figure 1

and an ovophile combining group. The spermatozoon is represented with a receptor capable of fitting the spermophile group of the fertilizin; the egg similarly contains receptors capable of fitting the ovophile group of the fertilizin. Within the egg represented as alternating with the egg-receptors, are also found certain molecules (anti-fertilizin) capable of uniting with the spermophile group of the fertilizin.

Union of a sperm receptor with a spermophile group of the fertilizin molecule activates the latter so that the ovophile group forms a union with an egg-receptor (fig. 1, sector 2, *a*); and I have postulated that the fertilizing action is the result of this union. But the activation of the fertilizin can by no means be regarded as confined to those molecules bound by the single penetrating spermatozoon. On the contrary, as we have seen that all the spermatozoa used in insemination bind only a minute proportion of the fertilizin contained in the eggs, it is necessary to assume that the activation of the fertilizin spreads beyond the point of attack of the successful spermatozoon; this is represented in figure 1, sector 2, *b* and *c*. Inasmuch as membrane formation is practically instantaneous all around the cortex, the activation of the fertilizin must spread with extreme rapidity; an activated molecule of the fertilizin must transmit the activated condition to its neighbor, in a fashion that may be compared by analogy to the spread of a stimulus. The assumption here is no different in principle from that which must be made in any theory of the fertilizing effect of the spermatozoon, and therefore, no new difficulty is introduced.

The postulated mechanism admits of five blocks in the process of fertilization viz.: 1. Absence of the fertilizin (fig. 1, sector 3); 2. Occupancy of the sperm receptors (fig. 1, sector 4); 3. Occupancy of the egg receptors (fig. 1, sector 5); 4. Occupancy of the ovophile side-chain of the fertilizin (fig. 1, sector 6); 5. Occupancy of the spermophile side-chain (fig. 1, sector 2, *b* and *c*). Of these we have examined and discussed in the body of this paper the first, fourth, and fifth, the first in the case of the long washed eggs, (p. 549) the fourth in the case of the inhibitor contained in the blood (p. 563), and the fifth in the case of the non-fertilizable

condition of the already fertilized eggs or those with membranes formed by other methods (p. 556). These cases have been sufficiently discussed already.

But there are two methods of blocking the mechanism according to the hypothesis, which we have not found, viz.: 2 (supra) the occupancy of the sperm receptors in such a way that these side-chains are no longer free to unite with the spermophile group of the fertilizin, which would result in loss of fertilizing power of the spermatozoon (fig. 1, sector 4); and 3 (supra), viz.: occupancy of the egg receptors, or molecules of the protoplasm with which the fertilizin reacts (fig. 1, sector 5). The discovery of these missing links in the theory would serve as strong confirmatory evidence of its essential correctness.

The latter of these two missing links remains purely hypothetical, but I think it is possible that the former may be represented by the phenomenon of the antagonism of sperm suspensions of different animal phyla as described first by Godlewski ('10 and '11) and subsequently by Herlant ('12, *a* and *b*).

These two authors give quite different interpretations of the results. While Godlewski, relying on the analogy of the antagonistic action of heterogenous hemolytic sera, regards the phenomenon as proof of Loeb's 'lysin theory' of fertilization, Herlant is more conservative, and believes that the explanation cannot be sought in any fundamental alteration of either sexual element; but is rather to be found in a purely 'humoral mechanism,' (i.e., in the effect of substance accompanying the spermatozoa) which he supposes to modify the physical state of the surface of the eggs in such way that the spermatozoa cannot penetrate. Herlant apparently overlooks the fact that penetration of the spermatozoon is not necessary for the cortical changes, as has been shown both by Loeb ('09 and '13) and myself ('12). There is nothing in the results of either author inconsistent with the idea, that the mechanism of inhibition in this case may be due to what I have called, "occupancy" of the sperm-receptors" as indicated in sector 4 of the diagram. This may be taken to mean merely such mutual action of the spermatozoa (or extractives thereof) on each other as to inhibit the union with the fertilizin.

This could readily be tested experimentally, for, in such a case, the mixed sperm suspension should no longer agglutinate on exposure to the fertilizin of the eggs used in the experiment. If, for instance, we were to find that sperm suspensions of *Arbacia* and *Nereis* were antagonistic in the sense of Godlewsky, so that mixtures of the two would not fertilize *Arbacia* eggs, then according to my hypothesis the *Arbacia* sperm in the inhibited mixture would not agglutinate with *Arbacia* fertilizin. The experiment yet remains to be performed.

## 2. THE TWO PHASES OF FERTILIZATION

In the second study of this series (Lillie '11) I showed that fertilization involves two phases in the case of the egg of *Nereis*; the first phase is accomplished before the penetration of the spermatozoon, and leads to changes in the cortex of the egg analogous to membrane formation in the sea-urchins. If the spermatozoon be then prevented from entering, the egg completes the maturation process only and does not segment. The cleavage of the egg is caused by some action of the spermatozoon after penetration. Loeb had also shown by entirely different methods the existence of two similar phases in the fertilization of the ovum of the sea-urchin.

The question, therefore, arises, does the mechanism of fertilization discussed in this paper deal only with the first, or with both phases of fertilization? To this it must be answered that the mechanism in question is primarily the mechanism of membrane formation. The described results demonstrate this so positively that farther discussion is superfluous on this point.

But the question remains whether there is any relation of such cortical changes to the internal phase of fertilization? Whether, for instance, if the spermatozoon could enter the egg without first forming the combination with the cortical fertilizin, it would then be in condition to carry out the second phase of fertilization, and cause segmentation of the egg? It has been shown, as is well known, that the egg can be induced to develop apparently without the cortical changes involved in membrane formation,

by the use of hypertonic sea-water for instance. It therefore seems possible that the two phases of fertilization may be disconnected in the case of the spermatozoon also, activated perhaps by different parts of it. On the other hand there is the possibility that fertilization is a continuous process and that the second step cannot proceed if the first be skipped; that the spermatozoon in other words receives some necessary modification, by union with the fertilizin or other substances experienced in the cortex of the egg.

The latter point of view seems more probable to me for the following reasons. In the first place I have made some yet unpublished observations in the case of *Nereis* which show that if the cortical changes be induced by artificial means there is a brief period in which insemination of the eggs may be followed by penetration of the spermatozoon, but without causing cleavage of the egg. Such a result might conceivably be due to a lack of synchrony between the movements of the egg-nucleus and the sperm-nucleus owing to the start given the egg-nucleus by the treatment prior to insemination. This explanation does not seem to me to be entirely reasonable, because, in *Nereis*, the sperm enters during maturation and has therefore abundance of time to prepare for union with the egg-nucleus. It seems more probable that the egg has lost something which renders the introduced sperm inefficacious. As a matter of fact, all the fertilizin is thrown off or neutralized in *Nereis* during the cortical changes, as I have determined by numerous experiments. Miss Allyn ('12) also determined for *Chaetopterus* that potassium chloride, which starts parthenogenetic development in this form, "initiates changes which increase with time and which are inimical to normal fertilization. They do not prevent the entrance of the sperm into the egg, but they prevent the normal behavior of the sperm in the egg." The cortical changes are not very obvious in *Chaetopterus*, but it is reasonable to suppose by analogy that in initiating parthenogenesis the fertilizin is bound; the sperm entering after this event are relatively inefficacious.

Dr. G. L. Kite kindly allows me to refer to some yet unpublished experiments performed last summer in which he injected,

by means of the Barber apparatus, from two or three to over twenty spermatozoa of the star-fish into eggs of the same species. There was never any indication of cleavage. The experiments were limited owing to interruption of Dr. Kite's work, so that the evidence in this case is not yet wholly conclusive; but the results indicate the necessity for normal penetration, if the spermatozoon is to be effective.

We therefore see a second function of the fertilizin, viz.: to prepare the spermatozoon for exercising fertilization effect in the interior of the egg. *The spermatozoon needs itself to be fertilized.*

De Meyer ('11) has observed swelling of the head of the spermatozoon under the action of egg-extractives, and I have described the same thing for Nereis ('13). But how such changes intervene in the internal fertilization reaction remains problematical. It is improbable that the swelling itself is significant; it is rather to be taken as indication of a more deeply seated physiological change which is essential for the completion of subsequent reactions.

### 3. THE LYSIN THEORY OF FERTILIZATION

Loeb's lysin theory of fertilization may be taken as the type of all theories which postulate that the spermatozoon produces and bears a fertilizing substance. Loeb holds that the spermatozoon causes membrane formation and thus initiates development by virtue of a lysin, and he holds this idea not because he, or some other, has isolated this substance and tested its action, but because the action of the spermatozoon in initiating development resembles the action of certain cytolytic substances, more especially, fatty acids, certain glucosides and the blood of some foreign species. Indeed he holds that all cytolytic agents induce membrane formation, and the action of the spermatozoon is regarded as merely a special instance. The spermatozoon, according to Loeb, introduces a second substance into the egg whose function it is to inhibit the harmful effects of membrane formation. However the effect of fertilization in increasing the rate of oxidation within the egg is due not to a catalyzer introduced by the spermatozoon but to the activation of catalyzers

already in the egg as shown by the fact that the rate of development is not increased by superposing fertilization on parthenogenesis or by polyspermic fertilization.

The evidence for the existence of a fertilizing lysin in the sperm is thus entirely analogical and would apply equally well to the conception that the sperm causes membrane formation by activating a substance in the egg, which would make the first part of Loeb's theory of fertilization consistent with the last part. Direct evidence is lacking for the lysin theory, but the whole series of facts described in this paper is direct evidence for the existence of the fertilizing substance in the ovum. We have obtained it in sea-water solution and we have studied its effects both on the spermatozoon and on the ovum and have found it to answer to all the necessary requirements. If the conclusions are accepted, we can hardly continue to believe in the existence of the hypothetical sperm-carried lysin.

But apart from this consideration, the lysin theory exhibits certain features of inadequacy. In the first place the failure of attempts to isolate such a substance from spermatozoa and obtain fertilization can be explained, as Loeb has pointed out, on the assumption that, though presumably present in sperm extracts, it lacks power of penetration in the isolated condition and needs the penetrative power of the living spermatozoon to carry it through the egg membrane. A similar assumption would have to be made with reference to the sperm receptors in my theory; so that this lacuna is not peculiar to the lysin theory.

In the second place the lysin theory affords no explanation whatever of the non-fertilizable condition of fertilized eggs. If membrane formation is the result of a superficial cytolysis produced directly by a lysin carried by the sperm and checked by operation of a second spermiatic substance effective after penetration of the spermatozoon, what is to prevent a second cytolysis by a second insemination? There is nothing in the lysin theory that affords the least explanation of this universal phenomenon: even concentrated sperm suspensions do not refertilize or exert a 'cytolytic' effect on fertilized eggs.

Loeb has noted that it is impossible to ascribe the increase of rate of oxidation after fertilization to the introduction of a cata-



lyzer by the sperm because polyspermy does not increase the rate of development over the normal. But he has neglected to note that the same argument applies to his lysin theory; if this theory were correct we should expect that polyspermy would increase the cortical cytolysis above the normal, and result in an unusually distended fertilization membrane; but this is not the case; membrane formation is not excessive, so far as observed, in polyspermic eggs. Yet the lysin theory requires that this should be the case.

It is also inconceivable that a lysin could act effectively in such high dilutions as the respective bulks of spermatozoon and ovum render necessary. Wilson estimates the bulk of the spermatozoon at about 1/400,000 that of the egg in the sea-urchin. The sperm head is not visibly diminished after entrance so it would be unreasonable to suppose that more than 10 per cent of its substance has been used in membrane formation. The lysin theory supposes that a spermatozoon cytolyzes a bulk of 4,000,000 times its lysin content! Not only so, but inasmuch as it is introduced at one point the lysin must diffuse with a speed and evenness that renders it effective simultaneously, and very quickly, at all points on the surface of the egg. This conception seems to me to be inconceivable, for the theory of lysin action presupposes a union molecule for molecule over the affected surface. We should also have a large spermatozoon for large eggs and a small spermatozoon for small eggs on the basis of a lysin theory, which is not the case.

These considerations are arguments not only against the lysin theory but also against any theory that presupposes that the spermatozoon is the bearer of a substance acting directly in causing formation of the cortical changes of fertilization.<sup>5</sup>

I may be allowed finally to point out that, even if some form of sperm extract should be shown to be effective in the production of the cortical change, such a result would be as consistent with the theory of indirect action or activation of an ovogenous substance as with the theory of direct action and would be no argument against the preceding considerations.

<sup>5</sup> Although Professor Loeb has committed himself very definitely to the lysin theory, he has not failed to note the theoretical possibility of the activation hypothesis (see Loeb 1913, p. 235; earlier reference given here).

## 4. THE THEORY OF PARTHENOGENESIS

A theory of parthenogenesis must also be a theory of fertilization, at least for the phenomena common to both. Similarly a theory of fertilization must be consistent with the facts of parthenogenesis. This has been generally recognized and it is one of Loeb's great services to have brought about the recognition of this principle and to bring the problem of fertilization definitely into the field of physiology. That the fertilizin theory is consistent in this respect is sufficiently obvious; indeed it makes the action of the sperm itself into a kind of parthenogenesis, for the sperm activates the fertilizing substance already present in the egg. The egg is self-fertilizing.

However, it lies beyond the province of this paper to consider the various methods of artificial parthenogenesis in detail and to examine the question of their consistency with the theory. It is obvious, I think, that the fertilizin theory from its very nature is more elastic with reference to this problem than any other, and is in general, therefore, more consistent with the established facts of the great variety of proved parthenogenetic agents.

Obviously it suggests certain lines of experimentation in parthenogenesis. Glaser has already followed one of these lines with some success, viz.: The effect of exposure of eggs to extracts or secretions of eggs of their own species; and he has ascertained that such extracts are rather efficient parthenogenetic agents in the case of *Arbacia*, especially when followed by treatment with hypertonic sea-water. He did not, however, observe the formation of membranes in eggs so treated, which leaves the mechanism of action rather obscure; farther analysis of the result is desirable.

But the whole procedure in experiments in parthenogenesis must take on a different aspect as soon as it is realized that the entire process, and not merely part of it, consists in acceleration of possible combinations in the egg, or increase in avidity of certain chemical groups.

It is not possible to separate the problem of fertilization from the general problems of cellular physiology; I may therefore be

allowed to point out the impossibility of regarding the interchange between the egg and its environment as controlled exclusively by diffusion through a semi-permeable membrane. However significant the rôle of permeability of the membrane may be, my experiments show that the egg cell is by no means bound in its exchange to diffusible substances; but that the membrane must be regarded as an active factor, and not only a passive one. Twenty years ago diffusion was assumed to play the chief rôle in the absorption from the intestine or in excretion by the kidney epithelium. Today we know that the excretion of urea means work by the kidney cell; it is an active, not a passive, process. Similarly the secretion of fertilizin by the egg cell, and possibly other cortical phenomena, must be regarded as active processes.

The ordinary chemical analysis of the cell begins with destruction of its more highly organized living constituents; it is obvious that such methods are inadequate for the investigation of the immediate reactions in living protoplasm. The results of these experiments may then gain a still broader interest if they may be taken to indicate a method of studying such reactions by the use of living cells as indicators.

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action which is dependent upon the change of permeability. Since anesthetics, as well as calcium and magnesium, render the plasma-membrane more resistant to the permeability-increasing action of salt-solutions,<sup>4</sup> they ought—if the above point of view is correct—also to prevent the cleavage-initiating action of these solutions. It was with this expectation that the following experiments were begun.

A well-marked antagonism of this kind was readily demonstrated in *Arbacia* eggs, although again as with the above anti-cytolytic action the anesthetics were found to be less effective than calcium or magnesium. The same anesthetics were used as in the preceding experiments with fertilized eggs,<sup>5</sup> in the concentrations there found just sufficient to prevent cell-division. The addition of anesthetics to the pure salt-solutions used (0.55 m KCNS and NaI) was found in many instances greatly to decrease the cleavage-initiating action of these solutions. Many of the eggs after exposure to the anesthetic-containing salt-solutions for five minutes remained to all appearance unchanged, while those treated similarly with the same salt-solution, free from anesthetic, all formed fertilization-membranes and underwent change of form or cleavage.<sup>6</sup>

The different anesthetics, however, were found to vary greatly in their ability thus to prevent the characteristic action of the salt-solution without injury to the eggs. Chloral hydrate and the urethanes proved much less effective in this respect than the alcohols, although, as shown in my paper on the influence of anesthetics on cleavage,<sup>7</sup> they are more favorable for anesthetizing the cleavage-process. The ability to counteract the salt-action thus need not run parallel with the power of suppressing cleavage. The case of potassium cyanide affords a further illustration of this difference in the influence of the same compound on these two processes. Cyanide does not appreciably interfere

<sup>4</sup> Loc. cit., 1912.

<sup>5</sup> Cf. Jour. Biol. Chem., 1914, vol. 17, p. 121.

<sup>6</sup> After-treatment with hypertonic sea-water is, of course, necessary to make such eggs develop to advanced stages.

<sup>7</sup> Loc. cit., 1914.

with the initiation of cleavage by the above salts, in spite of its powerful action in suppressing cleavage—a fact, it may incidentally be pointed out, indicating once more that this agent differs essentially in its mode of action from the anesthetics. That the normal course of the cleavage process is completely inhibited by anesthetics which only slightly counteract the salt-action probably indicates that a greater degree of resistance must be imparted to the plasma-membrane to prevent the action of the salt than to suppress cleavage; chloral hydrate and urethane are apparently less able than the alcohols to impart to the membranes the necessary stability or degree of resistance. The case of cyanide is different. Cyanide acts by directly inhibiting oxidations, and arrests cleavage through a quite different means from that exercised by the anesthetics, which appear to act primarily by altering the condition of the plasma-membrane and thus incapacitating this structure for the exercise of its normal activities.

Precisely how this effect is produced is undecided and requires further investigation; in some way—as I have shown in the experiments cited above—the presence of definite quantities of many lipoid-solvent anesthetics imparts an increased stability to the colloidal surface-films of cells and to other surface-structures such as cilia. Some change in the physico-chemical relations existing between the lipoid and protein components of the colloidal complex seems thus indicated. It has long been known that one colloid may exert a stabilizing influence upon another when the two are present together in solution. The lipoids and proteins of the plasma-membrane are possibly interrelated in some such manner;<sup>5</sup> if so, altering the condition of the lipoids will naturally affect the stability of the colloidal system, and under appropriate conditions (of temperature, concentration, etc.) will increase it. When this occurs, activities dependent on alterations of the plasma-membrane—especially the effects normally following changes of electrical polarization—are inhibited. The resulting state of temporary inactivity or irresponsiveness—what we call anesthesia or narcosis in irritable

<sup>5</sup> The observations of Lepeschkin are of much interest in this connection; cf. *Kolloid-Zeitschrift*, 1913, Bd. 13, p. 181.

tissues—appears to be the expression of this change in the physico-chemical properties of the plasma-membranes.

It was interesting to find that although anesthetics may thus inhibit the cleavage-initiating action of neutral salts, they have no appreciable influence on the similar action of the lipoid-solvent fatty acids. This observation has an intimate bearing on the general question of the mechanism of anesthetic action, as well as on that of the nature of the process underlying the initiation of cleavage. The fatty acid evidently acts by influencing the condition of the lipoids, and this effect is not prevented by anesthetics; the salt-action, on the other hand, which presumably affects all of the colloids and particularly the proteins of the membrane, is interfered with by these substances. It would thus seem that the salt and the fatty acid may produce the same effect by an action on different constituents of the plasma-membrane. I shall discuss the possible implications of this difference in the relation of anesthetics to these two cleavage-initiating agencies in the concluding section of this paper.

#### EXPERIMENTAL

##### *Influence of anesthetics on the cleavage-initiating action of neutral salts (KCNS, NaI)*

The procedure in these experiments was essentially as follows. The unfertilized Arbacia eggs were exposed for a brief period (four or five minutes) to the pure isotonic solution of the salt used (0.55 m KCNS or NaI), containing the anesthetic in known concentration; they were then returned to sea-water. Eggs treated in this manner with the pure salt-solution, free from anesthetic, form fertilization-membranes and cleave as described in my former paper; and if treated further with hypertonic sea-water a large proportion form blastulae. But of those eggs treated with the salt-solution containing a favorable anesthetic in the appropriate concentration a considerable and sometimes large proportion, varying with the anesthetic and time of exposure, remain to all appearance quite unaffected, showing no

sign of development or other change. If such eggs are fertilized, even after lying in sea-water for many hours (e.g., overnight) they develop into free-swimming larvae, showing that the eggs have not been essentially injured, but that the salt-solution has simply been prevented by the anesthetic from exerting its usual action. The degree of this preventive or antagonistic influence was estimated by comparing the respective proportions of eggs remaining thus unaltered after exposure to the pure and to the anesthetic-containing salt-solutions. In the majority of experiments eggs were also exposed—about fifteen minutes after the treatment with salt-solution—to hypertonic sea-water (100 volumes sea-water plus 16 volumes 2.5 M NaCl) for twenty minutes. The effect of the addition of anesthetics on the action of the hypertonic sea-water was also investigated. In most of the experiments the eggs were anesthetized in sea-water previously to being exposed to the anesthetic-containing salt-solution, i.e., were placed for about half an hour in sea-water containing the same anesthetic in the same concentration as in the salt-solution. The antagonistic influence of the anesthetic is found to be more pronounced after this preliminary anesthetization than when the eggs are transferred directly from normal sea-water to the anesthetic-containing salt-solution.

The details of the manipulation were kept as constant as possible. Equal quantities of eggs from the same lot were used in the different experiments of any series. The anesthetic-containing solutions were prepared shortly before using and kept in corked flasks. The brief exposure to the salt-solution was made in finger-bowls; the pure or anesthetic-containing sea-water was removed as far as possible and to the mass of eggs remaining (usually 2 to 3 cc.) a relatively large volume (usually 50 cc.) of the corresponding salt-solution was rapidly added; at the end of the four or five minutes of exposure a large volume (ca. 300 cc.) of sea-water was added and this was changed as soon as the eggs had settled, and again one or more times later. The sea-water in which the eggs were left after the final treatment was always changed several times to remove the last traces of anesthetic.



*Experiments with potassium thiocyanate*

Table 1 summarizes the results of two typical series of experiments. These illustrate in a typical manner the characteristic effects of magnesium and calcium in preventing the cleavage-initiating action of the alkali salt; the four alcohols also show a well-marked though less pronounced antagonistic action. The superiority of amyl alcohol and the relative ineffectiveness of ethyl urethane are also typical.

It will be noted that the degree of antagonism is decidedly greater in the case of those eggs (Series B) that were anesthetized previously to the treatment with the salt-solutions. This effect had been foreseen; presumably the preliminary anesthetization alters the plasma-membranes of these eggs, which are thus already in a relatively resistant condition when brought into the salt-solutions; hence the action of the latter on these eggs is more gradual than on eggs transferred to the solutions directly from normal sea-water. In the latter case it is to be assumed that the salt begins to exert its action before the anesthetic has had time to produce its full effect. There is a similar difference in the action of anesthetic-containing sodium chloride solutions on normal and on anesthetized *Arenicola* larvae.<sup>9</sup> Several other similar experiments gave the same general result.

The further fact appears clearly from these experiments that the anesthetics are less efficient than calcium and magnesium in counteracting the action of the alkali salt. A considerable and variable proportion of the anesthetic-treated eggs form fertilization-membranes and cleave, and later break down like eggs treated with the pure anesthetic-free salt-solution. The proportion of protected and intact eggs may, however, reach 80 or 90 per cent with a favorable anesthetic like amyl alcohol; such eggs appear quite normal and remain without change for many hours; if fertilized they develop into swimming larvae.

The physiological condition of these eggs is, however, not the same as that of normal eggs, but has been altered, apparently in the same general direction as in those eggs which form definite

<sup>9</sup> Cf. *Am. Jour. Physiol.*, 1912, vol. 29, p. 384.

fertilization-membranes and begin to cleave. This is true also, though to a less degree, of the eggs treated with the calcium- or magnesium-containing solutions. The effects of after-treatment with hypertonic sea-water show the difference between such eggs and normal unfertilized eggs. Hypertonic sea-water acting on normal eggs for this length of time has no apparent effect, causing neither membrane-formation nor cleavage. Its action on eggs previously treated with anesthetic-containing salt-solutions is, however, definite and well-marked. Even those eggs which, if left alone, show no signs of membrane-formation or other change, begin development, and a considerable proportion—though smaller than in the case of eggs with definite fertilization-membranes—reach the blastula stage. Many, of course, die before reaching this stage. Both the above series and that of table 2 afford numerous instances of this kind. It is clear that some physiological modification has been produced in the eggs, of the same nature as that which normally leads to membrane-formation, but insufficient by itself to produce this effect. The eggs are, however, brought into a condition in which they respond more readily to the action of the hypertonic sea-water. The antagonistic effect of the anesthetic is thus only partial; although no membranes are formed and there is no external sign of change, the eggs are rendered more responsive—or sensitized—to the subsequent action of the hypertonic sea-water. Examination of the above and succeeding tables will show that the proportion of eggs remaining unaltered after the treatment with hypertonic sea-water is always small. A certain proportion, however, do remain thus unaltered, apparently those in which the protective action of the anesthetic has been most complete. Such variability is always observed, though its precise basis cannot be defined as yet. Similar conditions are seen in the eggs treated with 0.55 m KCNS containing calcium or magnesium; these cations are decidedly more effective than the anesthetics, as shown above, and of the two magnesium has the greater action; always the great majority (>90 per cent) of eggs treated with these solutions remain to all appearance quite unchanged, and if fertilized the next day develop normally.

TABLE 1

August 13, 1913. In Series A the eggs were transferred directly from normal sea-water into the series of solutions of 0.55 m KCNS containing the anesthetics; they remained in these solutions 4 minutes, and were then returned to sea-water. In addition to the anesthetics,  $\text{CaCl}_2$  and  $\text{MgCl}_2$  were used for comparison. After the return to sea-water, part of the eggs remained without further treatment; another part were brought, about 15 minutes later, into hypertonic sea-water of the composition 100 volumes sea-water plus 16 volumes 2.5 m  $\text{NaCl}$ , where they remained for 30 minutes; they were then returned to sea-water. In Series B the treatment was in all respects similar, except that in each experiment the eggs were previously exposed for 30 minutes to sea-water containing the same anesthetic (and additional  $\text{CaCl}_2$  and  $\text{MgCl}_2$ ) in the same concentration as in the KCNS solution used in that experiment. From the anesthetic-containing sea-water each lot of eggs was transferred directly to its corresponding KCNS solution. The table describes the condition of the eggs after remaining about 18 hours in sea-water.<sup>2</sup>

## Series A

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTION ALONE	EGGS TREATED WITH SOLUTIONS FOLLOWED BY HYPERTONIC SEA-WATER
(1) 0.55 m KCNS	Practically all eggs are dead and cytolized	Majority (ca. 60 %) form blastulae
(2) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m $\text{CaCl}_2$	Practically all remain unaltered	Ca. 80 % remain unaltered; ca. 2-3 % form blastulae; the rest cytolized
(3) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m $\text{MgCl}_2$	Practically all remain unaltered	80-90 % remain intact; a few form blastulae (< 1 %); the rest cytolized
(4) 0.55 m KCNS + 2 vols. % n-propyl alcohol	Most are cytolized; ca. 15-20 % unaltered	Ca. 50 % form blastulae; very few intact; the rest cytolized
(5) 0.55 m KCNS + 1 vol. % n-butyl alcohol	Most are cytolized; ca. 30-40 % intact	Ca. 5 % remain intact; ca. 25-30 % form blastulae; the rest cytolized
(6) 0.55 m KCNS + 0.4 vol. % i-amyl alcohol <sup>1</sup>	Ca. 30-40 % intact; the rest cytolized	A few (ca. 5 %) intact; blastulae numerous (ca. 30 %); the rest cytolized
(7) 0.55 m KCNS + 0.015 % capryl alcohol	Nearly all cytolized; ca. 1 % intact	Ca. 50 % form blastulae; remainder cytolized
(8) 0.55 m KCNS + 1.25 % ethyl urethane	Almost all cytolized; little apparent effect	Large proportion form blastulae (ca. 75 %); the rest cytolized

TABLE 1—Continued  
Series B

	Same as Series A	Same as Series A
(1) 0.55 m KCNS	Practically no change; all eggs intact	A few blastulae (2-3 %); most eggs intact; some broken down
(2) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m $\text{CaCl}_2$	Practically all eggs intact	90 % or more remain intact; none form blastulae
(3) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m $\text{MgCl}_2$	Ca. 20-25 % intact; the rest cytolized	Few eggs intact; most cytolized; ca. 20 % form blastulae
(4) 0.55 m KCNS + 2 vols. % n-propyl alcohol	Ca. 35-40 % intact. The rest cytolized	Like 4B; a few intact; ca. 20 % blastulae
(5) 0.55 m KCNS + 1 vol. % n-butyl alcohol	Most eggs remain intact; (ca. 80-90 %)	A small proportion intact (ca. 5 %); ca. 20 % form blastulae; the rest cytolized
(6) 0.55 m KCNS + 0.4 vol. % i-amyl alcohol	Decided difference from 7A; ca. 30-40 % intact	Few intact (< 1 %); ca. 25 % form blastulae; the rest cytolized
(7) 0.55 m KCNS + 0.015 % capryl alcohol	A small proportion of intact eggs (ca. 5 %); the rest cytolized	
(8) 0.55 m KCNS + 1.25 % ethylurethane		

<sup>1</sup> The amyl alcohol used was iso-amyl (fermentation-amyl) alcohol,  $(\text{CH}_3)_2\text{CH}(\text{CH}_2)_2\text{CH}_2\text{OH}$ . In my former paper in this journal (1913, vol. 15, p. 23) this compound was inadvertently described as n-amyl alcohol.

<sup>2</sup> As a further test of the protective action of the anesthetics, spermatozoa were added the next morning (19 hours after the exposure to salt-solution) to the eggs that had been treated with the salt-solutions alone (without hypertonic seawater). A large proportion of the anesthetic-treated eggs formed blastulae; the number of eggs thus developing was higher in Series B than in Series A and showed a general correspondence with the results indicated in the first column of the table. Most of the eggs from Solutions 2 and 3 developed, and very few from Solution 8; the eggs from the other solutions showed intermediate conditions. A very small proportion of eggs treated with pure 0.55 m KCNS also survived and formed blastulae. The preliminary exposure to anesthetics in sea-water in Series B has no effect on the eggs; all remained unaltered next day and developed normally after fertilization.

Nevertheless, the after-treatment with hypertonic sea-water initiates development in a certain small proportion of these eggs, some of which form blastulae, though most stop short of this stage and undergo cytolysis. It is clear from the altered character of the response to hypertonic sea-water that some persistent modification has been produced by the previous treatment with salt-solution. What the nature of this modification is can only be surmised at present. The most probable general hypothesis seems to be that the plasma-membrane has been altered in some definite way—possibly rendered more permeable or more susceptible to alterations of permeability under changed external conditions. A changed state of electrical polarization would presumably accompany such a modified condition. On this view the increased responsiveness to hypertonic sea-water is analogous to the increased responsiveness of frogs' voluntary muscle which has been sensitized by brief exposure to isotonic solutions of (e.g.) sodium citrate or other sodium salt.<sup>10</sup> This form of sensitization is almost undoubtedly dependent on a surface-alteration, since it is produced within a few seconds by salts which either do not penetrate the normal plasma-membrane or do so with extreme slowness.<sup>11</sup>

The degree of protection afforded by the above alcohols may be decidedly greater than that shown in the above series. The following experiments (table 2) are especially favorable in this respect. These experiments also bring out clearly the effectiveness of anesthetics in inhibiting the characteristic action of hypertonic sea-water. Ethyl alcohol and phenyl urethane were used in addition to the anesthetics of table 1.

It will be seen from an examination of table 2 that the protective action of the alcohols is well-marked, while that of the urethanes, especially phenyl urethane, is comparatively slight. Amyl alcohol is distinctly superior to the others. These results are typical, as an examination of table 5 will show. A small proportion of the protected eggs remain unaffected by the after-

<sup>10</sup> Cf. J. Loeb, *Am. Jour. Physiol.*, 1901, vol. 5, p. 362.

<sup>11</sup> Cf. my paper in the *Proceedings of the Society for Experimental Biology and Medicine*, 1910, vol. 7, p. 170.

TABLE 2  
August 16, 1913. Unfertilized Arbacia eggs were exposed for about 28 minutes to sea-water containing the following anesthetics in the same concentrations as in the respective salt-solutions. From this sea-water each lot of eggs was transferred directly to its corresponding salt-solution:—0.55 m KCNS containing the anesthetic in the concentration given below. The exposure to the salt-solution was 4 minutes. About 15 minutes later part of the eggs were placed in hypertonic sea-water (100 volumes sea-water plus 16 volumes 2.5 m NaCl), another part (B) in hypertonic sea-water containing the same anesthetic in the same concentration as in the corresponding salt-solution. After 20 minutes in hypertonic sea-water the eggs were returned to normal sea-water. The condition of the eggs on the day following (after about 18 hours) is given in the table.

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTIONS ALONE		CONDITION OF EGGS TREATED WITH HYPERTONIC SEA-WATER	
			(A) free from anesthetic	(B) containing anesthetic
(1) 0.55 m KCNS	Practically all eggs are dead and cytolyzed		Ca. 50-60 % form blastulae; the rest are broken down	
(2) 0.55 m KCNS + 4 vols. % ethyl alcohol	Ca. 20-30 % remain intact; the rest cytolyzed		Ca. 50 % form blastulae; the rest are broken down	Most eggs are dead without signs of development; few (ca. 1 %) form blastulae. Ca. 4-5 % remain intact
(3) 0.55 m KCNS + 2 vols. % propyl alcohol	Ca. 25-30 % intact; the rest cytolyzed		Ca. 30 % form blastulae; a few 5 % remain intact; the rest broken down	Most dead; a few intact (ca. 5-10 %); ca. 2 % form blastulae
(4) 0.55 m KCNS + 1 vol. % butyl alcohol	Most eggs remain intact (ca. 60-70 %); the rest cytolyzed		Ca. 30 % form blastulae; a few intact (ca. 1 %); the rest broken down	Practically all are dead; no blastulae. Ca. 1 % intact
(5) 0.55 m KCNS + 0.4 vol. % amyl alcohol	Large majority (ca. 80-90 %) intact; the rest cytolyzed		Comparatively few blastulae cytolyzed. Ca. 5-10 % remain intact; the rest broken down	Many remain intact; ca. 30 % rest cytolyzed. No blastulae
(6) 0.55 m KCNS + 0.015 % capryl alcohol	Ca. 50-60 % remain intact; the rest cytolyzed		Ca. 20-30 % blastulae; ca. 5 % remain intact; the rest broken down	Most eggs cytolyzed; a few remain intact (2-3 %)
(7) 0.55 m KCNS + 1.5 % ethyl urethane	Protection relatively slight, ca. 10 % intact eggs		Large proportion of eggs form blastulae (> 50 %). The rest broken down	Nearly all dead without development; no blastulae; ca. 1-2 % intact
(8) 0.55 m KCNS + 0.06 % phenyl urethane	Little protective action; 1-2 % intact eggs; the rest cytolyzed		Blastulae numerous; ca. 60 % or more; the rest broken down	Nearly all are dead. A few form blastulae; ca. 1-2 % no intact eggs

treatment with hypertonic sea-water and remain intact the next morning; but the majority either undergo cytolysis or develop to a blastula stage. The proportion of eggs forming blastulae is in general smaller the greater the protective action, indicating that when the membrane-forming action of the salt is prevented the eggs respond less readily to the hypertonic sea-water; some entirely fail to respond.

It will be noted also that the presence of the anesthetic in the hypertonic sea-water greatly diminishes or entirely annuls the effectiveness of the after-treatment. Such hypertonic sea-water is not, however, entirely indifferent in its action, as is shown by the fact that relatively few eggs so treated remain intact; most undergo cytolysis and a few may develop. The same result appeared in six other series of experiments with hypertonic sea-water containing anesthetics. The favorable effect of the treatment is, however, almost entirely prevented by the presence of anesthetics in the protective or anesthetizing concentrations.

Removal of oxygen from hypertonic sea-water or the addition of cyanide also prevents its characteristic action, as Loeb found for *Strongylocentrotus*.<sup>12</sup> Cyanide has the same effect with *Arbaeia* eggs (cf. table 3). Loeb has interpreted these facts as indicating that some chemical process involving oxidations underlies the favorable action of the hypertonic sea-water. That this agent acts by modifying chemical processes in the egg is also indicated by the high temperature-coefficient of the times of exposure.<sup>13</sup> The fact that anesthetics have the same effect on the action of hypertonic sea-water as suppression of oxidations seems highly significant. Evidently the hypertonic sea-water induces some process of an oxidative nature; this process is checked or prevented by anesthetics. Now the anesthetics appear to act by altering the state of the lipid components of the plasma-membrane, thus rendering this structure more resistant to change than normally: it thus appears probable that the

<sup>12</sup> J. Loeb, *Biochemische Zeitschrift*, 1906, Bd. 1, p. 183.

<sup>13</sup> J. Loeb, *loc. cit.*; also University of California Publications, *Physiology*, 1906, vol. 3, p. 39.

hypertonic sea-water exerts its characteristic action primarily by changing the state of the plasma-membrane, and that the oxidative processes underlying the favorable effect of this after-treatment are a function of certain membrane-processes. We have thus further though somewhat indirect evidence that the plasma-membrane is a controlling factor in the intracellular oxidations.<sup>14</sup>

Chloral hydrate has little effect in preventing the action of 0.55 m KCNS. In five experiments with this anesthetic, in concentrations of 0.2 per cent to 0.1 per cent, the highest proportion of eggs remaining unaltered next day was *ca.* 10 per cent (table 5). Potassium cyanide showed no sign of protective action in any experiment. The series shown in table 3 illustrates the results obtained with these compounds.

It will be noted that although chloral hydrate and cyanide are almost without influence on the cleavage-initiating action of 0.55 m KCNS, both prevent entirely the favorable effects of after-treatment with hypertonic sea-water. This action was highly striking in the above series, since in every experiment the great majority of eggs formed blastulae after treatment with the pure hypertonic sea-water.

#### *Experiments with sodium iodide*

In experiments with this salt all the above alcohols showed well-marked protective action. The series summarized in table 4 will illustrate. The eggs were exposed to the freshly prepared 0.55 m NaI for four minutes; this is too brief an exposure to cause membrane-formation in all eggs, and about 20 per cent remained intact next morning; the proportion remaining intact was, however, much greater in the anesthetized lot, and fewer of these eggs formed blastulae.

A second similar series with five minutes' exposure to 0.55 m NaI, and containing in addition to the above alcohols ethyl urethane and chloral hydrate, gave similar results, although the protective effect was on the whole less pronounced. Urethane

<sup>14</sup> For more direct evidence of a relation of membranes to oxidations, cf. my recent paper in the *Journal of Biological Chemistry*, 1913, vol. 15, p. 237.



TABLE 3  
*August 28, 1913. The procedure was the same as in the series of table 2. The eggs were left in sea-water containing chloral hydrate and cyanide for 30 minutes before transfer to the salt-solutions. Exposure to salt-solutions was 4 minutes; to hypertonic sea-water, 20 minutes.*

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTION ALONE	CONDITION OF EGGS TREATED WITH HYPERTONIC SEA-WATER	
		(A) free from anesthetic	(B) containing anesthetic
(1) 0.55 m KCNS	Practically all eggs cytolyzed	Ca. 75 % form blastulae	None form blastulae; practically all cytolyzed
(2) 0.55 m KCNS + n/1000 KCN	Practically all eggs cytolyzed	Ca. 75-85 % form blastulae	None form blastulae; nearly all cytolyzed; a few intact
(3) 0.55 m KCNS + 0.2 % chloral hydrate	Almost all cytolyzed; a few intact eggs	Ca. 70-80 % form blastulae	None form blastulae; a few intact
(4) 0.55 m KCNS + 0.15 % chloral hydrate	Slight protective effect; nearly all cytolyzed	Majority form blastulae (> 75 %)	None form blastulae; a few intact
(5) 0.55 m KCNS + 0.01 % chloral hydrate	Little or no protective effect; practically all eggs cytolyzed	Same as above; ca. 75-80 % form blastulae	None form blastulae; a few intact

TABLE 4

August 29, 1918. The eggs were exposed to sea-water containing the following anesthetics for 30 minutes before transfer to the salt-solutions. The exposure to the salt-solutions was 4 minutes. After ca. 15 minutes in sea-water the eggs were transferred to hypertonic sea-water, with and without anesthetics, where they remained 20 minutes.

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTIONS ALONE	CONDITION OF EGGS TREATED WITH HYPERTONIC SEA-WATER	
		(A) Free from anesthetic	(B) containing anesthetic
(1) 0.55 m NaI	Most eggs are cytolyzed next day; ca. 20% remain intact	Ca. 30% form blastulae; ca. 10-15% remain intact	
(2) 0.55 m NaI + 4 vol % ethyl alcohol	Most eggs are intact next day (ca. 70%)	Blastulae are fewer than in Exp. 1 (ca. 10-15%); ca. 20% remain intact	Large proportion remain intact; >50% intact; a few blastulae; <1% the rest cytolyzed
(3) 0.55 m NaI + 2 vol % propyl alcohol	Most eggs remain intact (ca. 60-70%)	Blastulae few (ca. 5%); most eggs cytolyzed; small proportion intact	Most eggs cytolyzed; 10-15% intact; no blastulae
(4) 0.55 m NaI + 1 vol % butyl alcohol	Ca. 80% remain intact	Ca. 10% form blastulae; ca. 20% remain intact; the rest cytolyzed	None form blastulae; few (<5%) intact; the rest cytolyzed
(5) 0.55 m NaI + 0.4 vol % 1-amyl alcohol	Ca. 70-80% remain intact	Few blastulae (ca. 5%); ca. 5% remain intact	None form blastulae; ca. 5-10% remain intact; remainder cytolyzed
(6) 0.55 m NaI + 0.015% capryl alcohol	Ca. 50-60% remain intact	Few blastulae (ca. 5%); ca. 20% remain intact	None form blastulae; few intact (ca. 2-3%); the rest cytolyzed

was only slightly effective, while chloral hydrate showed well-marked action. In all cases the anesthetic prevented or greatly lessened the favorable effects of the after-treatment with hypertonic sea-water. The results with sodium iodide were thus in all respects similar to those with potassium thiocyanate.

In table 5 I have summarized the results of last summer's series of experiments with 0.55 m KCNS. The table includes the records of all experiments in which the eggs were exposed, previously to treatment with the anesthetic-containing salt-solutions to solutions of the same anesthetics in sea-water, in the manner already described.<sup>15</sup> The figures give the estimated proportion of eggs remaining intact the next morning after the treatment with the anesthetic-containing salt-solution alone. In all cases the proportion of eggs surviving the treatment with the pure 0.55 m KCNS (without anesthetic) was small—never more than 1 or 2 per cent.

The concentrations of anesthetic used in these experiments are those which just suffice to prevent cleavage in most fertilized eggs, without causing immediate injury.<sup>16</sup> The above solutions (with the exception of 2.4 v. per cent propyl alcohol and 1.2 v. per cent butyl alcohol, which are too strong) are thus, as regards their inhibiting effect on cleavage approximately equivalent. Nevertheless in their ability to prevent the cleavage-initiating action of salt solutions they show marked inequalities. The alcohols are greatly superior to chloral hydrate and the urethanes; amyl alcohol is distinctly more effective than the others, while butyl alcohol appears somewhat more favorable than propyl or capryl, and ethyl is only moderately effective. This lack of parallelism between the anesthetic and the above protective actions may seem inconsistent with the general hypothesis advocated in this paper. But when the essential dissimilarity between the process of normal cleavage and the external action of a pure salt-solution is considered, the discrepancy ceases to be surprising. That

<sup>15</sup> With the exception of a few experiments in which the results of fertilization showed that the eggs were defective.

<sup>16</sup> Cf. my paper on the influence of anesthetics on cleavage cited above, pp. 128



a given anesthetic may interfere with one set of processes and not with another has long been known. It would appear rather, as suggested above, that while chloral hydrate and the urethanes impart to the plasma-membranes a degree of resistance sufficient to prevent the normal changes of cleavage, this resistance is insufficient to prevent the relatively violent action of the pure salt-solution. The alcohols, for some reason as yet obscure, protect the egg more effectively against the latter action. They are, however, quite ineffective in preventing the cleavage-initiating and cytolytic action of fatty acids. The ability of an anesthetic to suppress or prevent a given process thus depends both on the nature of the anesthetic and of the process itself. The experiments with fatty acid about to be described will illustrate this.

*The cleavage-initiating action of fatty acids in the presence of anesthetics*

The above anesthetics entirely fail to interfere with the formation of fertilization-membranes or the initiation of cleavage by fatty acids. In a number of experiments, conducted similarly to those already described, in which the parthenogenetic agent was sea-water containing acetic or butyric acid (2 to 3 cc.  $\frac{N}{10}$  fatty acid plus 50 cc. sea-water) the results were entirely negative. The addition of the anesthetic seems indeed to increase the injurious action of the acid. Chloral hydrate is equally ineffective. The urethanes were not tried. The following series with the alcohols will illustrate (Table 6).

From these experiments it is clear that the above anesthetics are completely unable to counteract the action of the fatty acid. Their antagonistic influence seems to be confined to the salts. Experiments with sea-water containing additional calcium and magnesium chloride (without altering the osmotic pressure) gave an analogous result. It was thought that possibly by increasing the proportion of these salts in the medium the eggs might be rendered more resistant to fatty acid. The following solutions were used: 100 volumes sea-water *plus* respectively

TABLE 6

August 20, 1913. The eggs were exposed to the solutions of the following alcohols in sea-water for 28 minutes. They were then treated for 2 minutes with sea-water containing 2% butyric acid (50 cc. sea-water plus 2 cc. 5% butyric acid). After an interval of 15 minutes part were exposed to hypertonic sea-water, with and without anesthetics, for 20 minutes. The condition of the eggs after 19 hours was as follows:

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTIONS ALONE	CONDITION OF EGGS AFTER TREATED WITH HYPERTONIC SEA-WATER	
		(A) without anesthetic	(B) containing anesthetic
(1) Sea-water + 2.5% butyric acid	All cytolyzed	80-90% form blastulae	Nearly all cytolyzed; a few blas-
(2) Same + 4 vol. % ethyl alcohol	All cytolyzed	Blastulae fewer than in Exper. 1;	tulac: < 1%
(3) Same + 2 vol. % n-propyl alcohol	All cytolyzed	ca. 60-70% Most eggs cytolyzed; ca. 5% form blastulae	All cytolyzed
(4) Same + 1 v. % n-butyl alcohol	All cytolyzed	None form blastulae; all cyto-lyzed	All cytolyzed
(5) Same + 0.4 v. % i-amyl alcohol	All cytolyzed	None develop; all cytolyzed	All cytolyzed
(6) Same + 0.015% capryl alcohol	All cytolyzed	Many form blastulae; ca. 50% the rest cytolyzed	Nearly all cytolyzed; one or two blastulae seen

10, 20 and 50 cc. 0.35 m  $\text{MgCl}_2$ , similar mixtures of 0.35 m  $\text{CaCl}_2$  and sea-water, and mixtures containing both salts; 2 cc.  $\frac{N}{16}$  butyric acid was added to 50 cc. of each solution, and the action on unfertilized eggs was tested as above. The results again were entirely negative as regards protective action: in all cases two minutes' exposure to these solutions was followed by cytolysis. After-treatment with hypertonic sea-water proved ineffective in all cases, none of the eggs so treated forming blastulae (with the exception of a few from 100 volumes sea-water plus 10 volumes 0.35 m  $\text{MgCl}_2$ ). The presence of an excess of calcium or magnesium in the sea-water thus prevents the eggs from developing favorably later, although it does not hinder the cytolytic action of the fatty acid.<sup>17</sup>

<sup>17</sup> Experiments, with unfertilized eggs conducted in the summer of 1911 on the antitoxic action of  $\text{CaCl}_2$  on isotonic  $\text{NaCl}$  solutions containing acetic acid gave entirely negative results. The following series will illustrate. Eggs were left for two hours in the solutions, then returned to normal sea-water and fertilized. The results were as follows (condition of the eggs next day):

Solution	Result
(1) Pure 0.55 m $\text{NaCl}$ .....	ca. 40-50 % form blastulae
(2) 95 vols. 0.55 m $\text{NaCl}$ + 5 vols. $\frac{M}{2}$ $\text{CaCl}_2$ .....	all form blastulae
(3) 0.55 m $\text{NaCl}$ + $\frac{N}{200}$ $\text{CH}_3\text{COOH}$ .....	all eggs dead
(4) 0.55 m $\text{NaCl}$ + $\frac{N}{200}$ $\text{CH}_3\text{COOH}$ + $\frac{M}{40}$ $\text{CaCl}_2$ .....	all dead
(5) 0.55 m $\text{NaCl}$ + $\frac{N}{400}$ $\text{CH}_3\text{COOH}$ .....	all dead
(6) 0.55 m $\text{NaCl}$ + $\frac{N}{400}$ $\text{CH}_3\text{COOH}$ + $\frac{M}{40}$ $\text{CaCl}_2$ .....	all dead
(7) 0.55 m $\text{NaCl}$ + $\frac{N}{800}$ $\text{CH}_3\text{COOH}$ .....	all dead
(8) 0.55 m $\text{NaCl}$ + $\frac{N}{600}$ $\text{CH}_3\text{COOH}$ + $\frac{M}{40}$ $\text{CaCl}_2$ .....	all dead
(9) 0.55 m $\text{NaCl}$ + $\frac{N}{800}$ $\text{CH}_3\text{COOH}$ .....	all dead
(10) 0.55 m $\text{NaCl}$ + $\frac{N}{800}$ $\text{CH}_3\text{COOH}$ + $\frac{M}{40}$ $\text{CaCl}_2$ .....	all dead

Thus  $\text{CaCl}_2$  in concentrations which completely prevent the toxic action of the  $\text{NaCl}$  solution has no effect on the toxic action of acetic acid.  $\text{CaCl}_2$  also showed no antitoxic action in 0.55m  $\text{NaCl}$  containing  $\text{NH}_4\text{OH}$  in concentrations from  $\frac{N}{40}$  to  $\frac{N}{2560}$ . It also failed to antagonize  $\text{HCl}$  in concentrations from  $\frac{N}{160}$  to  $\frac{N}{3200}$ ; but with weaker solutions ( $\frac{N}{3400}$  to  $\frac{N}{25600}$   $\text{HCl}$ ) some antitoxic effect was seen. Arbacia eggs thus differ from *Fundulus* eggs, which are protected by salts to a considerable degree against injury by acetic acid in  $\frac{N}{333}$  concentration (cf. J. Loeb, *Biochemische Zeitschrift*, 1912, vol. 47, p. 151). *Fundulus* eggs are, however, surrounded by a resistant chorionic membrane.

## CONCLUSIONS

We reach thus the general result that the formation of fertilization-membranes and the initiation of cleavage may be prevented by anesthetics when the parthenogenetic agent is a neutral salt, but not when it is a fatty acid. This contrast is what would be expected on the assumption that the essential action of the anesthetic is superficial, and consists in rendering the plasma-membrane more resistant to alterations of permeability. Hence the salt, which does not readily penetrate the unaltered egg and produces its effect by increasing the permeability of the plasma-membrane, is rendered less effective when the membrane has been rendered relatively resistant or stabilized by the anesthetic. The fatty acid, on the other hand, which penetrates the plasma-membrane readily under all conditions, by virtue of its lipid-solubility, is not prevented in its action by anesthetics.

Fatty acids and neutral salts represent two classes of agents, one of which penetrates the egg-surface readily, the other with difficulty and apparently only after increasing the permeability of the plasma-membrane. Both induce parthenogenesis in a typical manner. It is significant that of these two parthenogenetic agents one should be influenced in its action by anesthetics, the other not. The fact that it is the more penetrating of the two which is uninfluenced seems to indicate that the agent produces its essential effect by acting on some portion of the egg-cytoplasm situated *within* the most external surface-layer, and that calcium and anesthetics inhibit the action of salt-solutions because they prevent the access of the salt to this critical region of the egg. Otherwise it is difficult to understand why the anesthetic, which apparently stabilizes the surface-layer and hinders alteration of permeability, is without influence on the action of the fatty acid, although it inhibits the action of the salt. The entrance of the salt but not of the lipid-soluble fatty acid would be hindered by agents which act (like anesthetics and Ca salts) by preserving semi-permeability unaltered, since semi-permeability relates to lipid-insoluble substances<sup>18</sup> only, as Overton first showed.

<sup>18</sup> I.e., non-colloidal substances. Ruhland has shown that various dyes which form colloidal solutions are exceptions to Overton's general rule. Cf. Jahrb. wiss. Botanik, 1908, Bd. 46, p. 1.



It is of course also possible that the actual entrance of the salt into the egg is unnecessary, and that a purely superficial action sufficient to increase permeability to a critical degree and thus cause a definite depolarization-effect is all that is necessary. There is at present no certain means of deciding between these two alternatives. If the salt increases permeability to a sufficient degree, it will naturally enter the egg and produce certain effects in its interior. It is, however, clear that there is nothing specific about the salt-action; all that is needed is that it should be sufficiently energetic. The entrance of special substances from outside into the egg is not necessary for parthenogenesis: the effects of temporary warming and mechanical agitation upon starfish eggs are a sufficient proof of this. On the other hand, certain substances as fatty acids and lipoid-soluble alkalis, do undoubtedly produce their effects by penetrating the egg.<sup>19</sup> The comparative ineffectiveness of the lipoid-insoluble and non-penetrating alkalis and acids indicates this clearly. The most probable conclusion seems to be that the same effect can be produced by a purely superficial action, like that of a salt or the electric current, as by one operating at some region within the interior of the egg.

There are many indications that the primary effect in the activation of the egg—whether by the spermatozoön or a parthenogenetic agent—is superficial and consists in an alteration of the surface-layer of protoplasm—the region somewhat vaguely designated as plasma-membrane. The term 'plasma-membrane' in its application to the surface-film seems at present to require more precise definition. The conception of this structure as a thin homogeneous haptogen membrane exercising passive mechanical and osmotic functions is clearly inadequate. It must rather be regarded as essentially a superficial portion of the living protoplasm, characteristically modified in its composition and physical properties by surface forces. We must thus ascribe to it a characteristic chemical organization and metabolism as well as the characteristic physical and other properties, such as

<sup>19</sup> Cf. Loeb's recent paper on "The comparative efficiency of weak and strong bases in artificial parthenogenesis," *Jour. Exp. Zool.*, 1912, vol. 13, p. 577.

selective semi-permeability, that have led to its distinction from the more internal protoplasm. Conceived in this way, it corresponds closely to what morphologists designate as the cortical region of the egg, or at least to the most external layer of this region.

Changes in this region form a highly characteristic accompaniment of fertilization in many if not in all eggs; associated with these changes is a marked temporary increase in the general permeability of the surface-layer. The relation of these surface-changes or cortical processes to the initiation of cell division and development is evidently a critical one.<sup>20</sup> Once they are accomplished the developmental mechanism, hitherto held in check, resumes operation and—provided external and other conditions are favorable—continues its course automatically to the adult stage. It is clear, from the diversity of the conditions that may initiate development, that some process specific to the egg and quite independent of the nature of the activating condition forms the primary event in fertilization. The spermatozoon or the parthenogenetic agent in some way removes the hindrance to this process. What the nature of the latter is may be partly inferred from the results of recent experiments on the physiology of fertilization. The observations described in this paper support the view that some change in the cortical region of the egg-protoplasm, beneath the most external semi-permeable layer of plasma-membrane proper, forms the initial stage of the fertilization-process. The immediate surface of the egg has semi-permeable properties relatively to most water-soluble lipoid-insoluble substances, and apparently must undergo increase of permeability in order that such a salt as NaI or KCNS may produce its characteristic effect. As already said, it is uncertain whether the salt acts by entering and then affecting directly the state of the colloids in the cortical region, or whether it acts without entrance, possibly by altering the electrical polarization of the plasma-membrane. It seems probable, however, from the general effectiveness of lipoid-alterants, that some

<sup>20</sup> Cf. F. R. Lillie, on The cortical changes in the egg of Nereis. *Jour. Morph.*, 1911, vol. 22, p. 361.

change in the condition of the lipoids—possibly in the inter-relations between lipoids and proteins—is the primary effect produced and that this change then initiates some specific chemical reaction which determines directly or indirectly the characteristic surface-changes of fertilization, namely, secretion of cortical material, formation of fertilization-membrane, temporary change in osmotic properties of the plasma-membrane with accompanying electrical depolarization.

This view emphasizes the analogy between the activation of the resting egg and the general process of stimulation.<sup>21</sup> In stimulation the primary event is a depolarization of the limiting membrane; similarly in the fertilization-process the electrical variation accompanying the above surface changes forms most probably the critical or 'releasing' event on which the rest of the process automatically follows. One gains the impression that in the resting egg-cell, as well as in the resting muscle or nerve, certain substances are hindered from interacting by the electrical polarization at the cell-surface; just as in a battery with open circuit the chemical reactions on which its operation depends are held in check by the polarization at the surface of the plates;<sup>22</sup> that is, the passage of ions into or out of solution is thus prevented and with it all effects, chemical and other, dependent on the flow of electricity through the circuit. Under analogous conditions in the living cell a brief depolarization might suffice to release the impediment to the chemical interaction forming the primary event in the response—whether to stimulation or (in the case of the egg-cell) to fertilization. It is noteworthy that in many if not in most irritable cells the response is specific and constant and independent of the character and intensity of the stimulus; that is, recent research indicates that the "all or none" law applies to irritable elements in general,<sup>23</sup> and not only to heart-muscle, and it may be said

<sup>21</sup> For a fuller discussion of this analogy, cf. my recent paper in the *Journal of Exp. Zool.*, 1913, vol. 15, p. 23.

<sup>22</sup> The solution-tension of the ions being compensated by the electrostatic attraction between the plate and the oppositely charged adjoining layer of solution.

<sup>23</sup> Cf. especially the recent articles from the Cambridge Physiological Laboratory by Lucas and Adrian in the *Journal of Physiology*.

with some qualification to apply also to the resting egg-cell. The removal of an inhibiting condition, whatever the means employed, is the essential requirement for fertilization. What follows is determined entirely by the nature of the egg itself.

The recent work of F. R. Lillie<sup>24</sup> reinforces still further this general point of view. His results indicate that in fertilization a union of a specific amboceptor-like substance, contained in the egg-cortex, with some other specific substance, also furnished by the egg, forms the primary or determinative event. The spermatozoon acts by removing the hindrance to this interaction, but other agents may act similarly—hence the possibility of parthenogenetic fertilization. This conception makes it clear why the presence of the sperm is unnecessary to the activation of the egg, and suggests that in its activating capacity this structure plays essentially the part of a specific releasing mechanism, in a manner which is thus closely analogous to that of a stimulus, as already indicated. The ensuing developmental processes are specific to the egg under investigation and require special analysis in each case.

#### SUMMARY

The chief experimental results and general conclusions of this paper may be briefly summarized as follows:

1. The action of pure isotonic solutions of neutral salts (0.55 m KCNS, NaI) in inducing formation of fertilization-membranes and cleavage in the unfertilized eggs of *Arbacia* may be prevented by anesthetics as well as by calcium and magnesium salts. The effective concentrations are those which just suffice to prevent cleavage in fertilized eggs.

2. The anesthetics are less effective than calcium or magnesium, and vary characteristically in effectiveness. The monohydric alcohols of the aliphatic series are the most favorable of those tried; the order of relative favorability runs: i-amyl > n-butyl > n-propyl and capryl > ethyl. Phenyl and ethyl urethanes have comparatively slight action, and chloral hydrate still less. Cyanide is ineffective.

<sup>24</sup> Cf. Science, N. S., 1913, vol. 38, p. 524.

3. The anesthetics have no inhibiting influence on the cleavage-initiating action of fatty acids. Since fatty acids readily penetrate the unaltered plasma-membrane, while salts do not, and since both agents are equally effective in inducing parthenogenesis, this difference indicates that the parthenogenetic agent acts at some point within the most external layer of the egg.

4. The favorable effects of after-treatment with hypertonic sea-water are prevented by anaesthetics as well as by cyanide. This result indicates that hypertonic sea-water, as well as anesthetics, acts by modifying the condition of the plasma-membrane.

# OLFACTORY REACTIONS IN AMPHIBIANS

JONATHAN RISSE

ONE FIGURE

## CONTENTS

Introduction.....	618
Food and odors.....	618
1. Relation of odors to food.....	618
2. Materials and methods.....	619
3. Experiments.....	620
4. Experiments with non-living food.....	624
Food and darkness.....	625
1. Materials.....	625
2. Experiments.....	626
3. Abnormal odors and darkness.....	627
Odor-stream experiments.....	628
1. Apparatus.....	628
2. Rate of flow of odor-stream.....	630
3. Methods.....	630
4. Substances used in the tests.....	631
5. Experiments.....	631
6. Controls and operations.....	634
Experiments with tadpoles.....	638
1. General.....	638
2. Methods.....	639
3. Experiments.....	640
a. First set of tadpoles.....	640
b. Second set of tadpoles.....	641
c. Third set of tadpoles.....	641
Discussion.....	645
1. Toads.....	645
2. Tadpoles.....	647
3. Conclusion.....	648
Summary.....	649
Bibliography.....	651

## INTRODUCTION

It is the purpose of this paper to record some observations and experiments on the olfactory reactions in certain amphibians, more especially in the toad (*Bufo americanus* LeConte). Initial experiments were tried on frogs (*Rana virescens* and *R. catesbiana*), both larval and adult, but the work did not prove promising and was therefore given up.

These investigations were undertaken at the suggestion of Dr. G. H. Parker of the Zoölogical Laboratory of Harvard College and to him for his kindly interest and helpful advice I wish to express my deepest appreciation.

## FOOD AND ODORS

*1. Relation of odors to food*

There is little evidence that the relation of odors to food has been taken into account in any investigation of the habits of the different amphibians. In the quantitative studies of the stomach contents of frogs and toads no evidence has been found to show that certain foods are preferred by these animals. Fischer-Sigwart ('97) believed that frogs and toads were indiscriminate feeders. Needham ('05) has shown the food of the bullfrog (*R. catesbiana*) to be extremely varied. Lockwood ('83) in speaking of the toad, says "I do not believe it can smell. It catches insects, but only when such probable food is in motion." Knauer ('75), however, mentions cases where decomposing animal food was rejected by toads after having been taken into the mouth. Schaeffer ('11) found that certain caterpillars were refused by frogs in a similar manner. In both cases other factors may have been of disturbing influence. Hartman ('06) found no special preponderance of one species over another in the insects taken from the stomach of toads collected at random. Garman ('92) has records of the food of toads from which one might conclude that ants were more sought after than other insects. Such a condition, however, may have been due to the fact that younger newly metamorphosed toads being close to the ground met ants more frequently than they did.

other insects. Hodge ('98) refers to the large numbers of house-flies eaten by the toad. Schaeffer ('11) mentions the variety of insect food taken by frogs in confinement. Quaintance and Brues ('05) show how toads make use of very diverse forms of insects as food. Slonaker ('00) and others have fed meat to toads, by simulating the motion of small insects to attract attention. The behavior of toads and frogs in confinement leads to the inference that food ordinarily must be in a living condition and in motion to be attractive.

Among food materials taken by the toad under normal circumstances there are many insects with characteristic odors. Conradi ('01) infers that the odor of the cucumber beetle (*Anasa tristis*) is inimical to the toad, but Hill ('73) observed no disastrous results to toads that had fed on this insect. Neither frogs nor toads hesitate to make use of other vertebrates as food, when occasion offers, or to devour members of their own species. Can it be shown that these animals are stimulated to seek for food or refuse it because of odors? With this question in mind the following feeding experiments were carried on with the toad (*Bufo americanus* LeConte).

## 2. Materials and methods

The toads used in these experiments were obtained in the vicinity of Cambridge, Massachusetts, quite late in the year. They were kept in a large box containing soil and leafmold in a moderately cool basement room. The soil was kept damp and the box dark. Some of the animals buried themselves in the soil, others took shelter under bits of wood. The animals reacted normally in all respects, taking food when offered. The food consisted mainly of mealworm larvae (*Tenebrio molitor*), of earthworms and dungworms (*Allophora foetida*), flies and other insects. Some of the toads were removed to large jars for greater convenience. Not all the animals were alike, some showing a greater tendency to hibernate than others. Food was generally given the toads at intervals of several days, but this procedure was modified as necessary. For convenience the experiments were carried on with the



toads confined in a large metal pan. In this way the animals were kept within bounds but were still allowed much freedom of movement.

### *3. Experiments*

The first experiment was made to determine whether choice was made between two forms of food with specific odors. Previous to the trials earthworms had been fed freely to the toads, and it might be supposed that because of the peculiar odor of the dungworm, the latter would be refused as food.

Toads No. 1 and No. 2 were in the box in which the experiments were conducted before the dungworms were introduced. Toad No. 1 saw the worms soon after they had begun to crawl. It quickly hopped toward the worms and attempted to snap up the nearest worm. The attempt was unsuccessful and the lips were wiped with the forefeet. Sandgrains on the lips evidently produced a mechanical stimulus which was followed by the wiping.

The dungworms were then freed of all the adhering sand and again placed in the center of the box. Although the movement of the worms was sufficient to attract the toads, no further attempt was made to take the worms. When a mealworm larva was put in with the dungworms, it was quickly snapped up by the nearer toad, No. 1.

Dividing a dungworm then into a number of pieces, these were placed in a shallow vessel before the toads. Into the vessel there were also dropped two mealworm larvae. From the mass of wriggling pieces one of the mealworms was immediately selected and swallowed. Upon the dorsal surface of the other mealworm a drop of oil of pennyroyal was now placed; the mealworm with the oil was also quickly taken. Toad No. 2 made several unsuccessful attempts to take the remaining dungworm, but abandoned the dungworm, taking later some mealworms that had been put in the box. Similar trials with two other toads gave like results, leading to the conclusion that the dungworm is not used as food under normal condition, a conclusion that was subsequently shown to be false.

Some days later, the same toads, Nos. 1 and 2, were again tested with the dungworms. Three worms of medium size placed in the center of the box were immediately noticed by Toad No. 1. No attempts to take any worms followed. The odor characteristic of the worms was evident and the cutaneous exudate was visible. A mealworm dropped into the vessel among the dungworms quickly became covered with the slime of the worms. That the mealworm was recognized as a new object by the toad, is proven by the actions of the toad. The attitude peculiar to the animals when watching some object, was immediately assumed. As soon as the mealworm had crept away from the dungworms, the toad snapped it up. Another mealworm dropped into a dish containing some fragments of dungworm was watched by the toad as long as it moved. After it ceased moving it was of no more interest to the toad. No attention was paid to the fragments of dungworm although they were wriggling. A second mealworm smeared with the exudate from the earthworm was quickly snapped up by No. 1. This reaction was followed by the wiping of the lips with the forefeet; whether this was done to remove the slime adhering to the lips was not determined. The wiping action again took place when the same toad took another mealworm similarly prepared. Toad No. 2 exhibited the same reactions under similar conditions.

A series of trials with the different toads proved that fragmented dungworms were never taken, even though they exhibited decided movements. The trials also showed that the toads preferred the mealworms, and that the odor of the dungworms when applied to the mealworms was not deterrent to the toads.

Trials carried out with the same toads, to determine their preference for mealworms over earthworms, indicated that the mealworms were the more desired form of food. These were selected from among the earthworms. It is not clear why this should have been so; previously and later in the course of the experiments both earthworms and mealworms were taken promiscuously.

## 2. Experiments

To ascertain whether any stimulus other than the optic is called into play in the finding and taking of food the following experiments were performed.

Toad No. 1 was placed in the box, which was empty except for a covering of moist filter paper on the floor, and allowed to remain undisturbed for some time. There was no excited hopping about during this period of illumination. The light having been cut off, the toad crept along the wall, and finally came to rest in a corner where it attempted to burrow as though it were on soil. After two hours of darkness some mealworm larvae were introduced into the box. These were placed centrally on the floor and as these changes were made in darkness the larvae were not seen by the toad. The toad was then lifted from its position in the corner of the box and placed immediately over the glass plate on which the larvae were and facing them. In this position there was no thigmotactic stimulation, as there would have been in the corner position. By listening to the sounds within the box the movements of the toad could be followed. The position of the larvae and the toad were noted at the end of two minutes of darkness. The toad had resumed the corner position, the larvae had moved out and away from the plate, one of them being within 2 inches of the toad. Two minutes of darkness brought no further movement on the part of the toad. The larvae were distributed around the sides of the box. After another minute of darkness, the light was turned on and at the first visible movement of the mealworms, the toad turned and jumped toward them. There followed no movements on the part of the toad in successive periods of darkness; but each time the box was illuminated the least movement on the part of the mealworms called forth characteristic movements from the toad.

Toad No. 2 was tried with dungworms in a manner similar to that just described for Toad No. 1. No notice was paid to the worms in the dark but in the light the toad followed the

worm some distance and finally picked it up, using both the tongue and lips to do so.

In another trial, the toad was placed in the center of the floor and the worms were distributed in the portions of the box near the walls. The illumination was lateral, thus leaving one side of the box quite dark. Movement of worms in the darker regions of the box were not seen by the toad, even when it was turned toward the worms. When a worm moved in the light part of the box, the movement soon attracted the attention of the toad and the characteristic reaction followed immediately. Repetition of the experiment several times with other toads gave the same results.

When the dungworms were replaced by pillbugs (*Oniscus*), these were immediately caught when in the light, but not when in the dark.

Other trials with mealworms, earthworms and dungworms gave like results; only during the periods of illumination was the food sought and then with no discrimination in favor of any particular form.

Other trials, with very dim daylight as a means of illuminating the chamber gave identical results. If the mealworms or the dungworms were in the darker portions of the chamber, they were unnoticed by the toad. When the box was covered so as to cut off all illumination, there were no movements on the part of the toad; darkness prohibiting entirely any food seeking activities.

In the lighted portions of the chamber the worms were only seen if in the direct visual field; if somewhat to the rear or to the side of the toads they apparently could not be seen, except when in very vigorous motions.

### *3. Abnormal odors and darkness*

In connection with the darkness experiments, trials were made with the same odorous substances that had been used in the preceding experiments. The same individual toads were used as before. Mealworms were treated with applications of oil of clove, carbon bisulphide, and oil of rose gera-

nium' and were allowed to remain in the experimental chamber with the toads under the same light conditions as described in the preceding trials. The presence of the odors did not stimulate the toads to seek or avoid the worms in the dark. Again, as observed in the previous trials, the presence of the odorous substance did not deter the toads from taking food in the light.

#### ODOR-STREAM EXPERIMENTS

##### *1. Apparatus*

From the experiments previously recorded there was no positive evidence that odors were concerned with the taking of food by the toads. Odorous substances when taken into the mouth by accident do not stimulate the receptor organs to the degree of inhibiting the act of deglutition. A possible explanation for this might be sought in the relation of the external nostrils to the mouth. Even if the olfactory function were present to but a slight degree, it might be possible to demonstrate its presence by leading the odors directly to the nasal opening. If the vapors could be brought directly to the epithelial surfaces in appropriate manner, reactions might occur. It seemed desirable to devise some method for doing this.

After some trials, an apparatus of a satisfactory kind was finally devised. An outline of it is shown in figure 1. The arrangement of the parts is as follows: From the reservoir, *A*, water displaced the air contained in *B*. By means of screw clamps at *x* and *y* the rate of flow of the water was controlled. The clamp, *x*, was adjusted for the flow, while clamp *y* is used for starting and stopping the stream as a whole. *C* is a small reservoir in which was suspended a vial, *D*, containing the odorous substance used. The distal end of the outlet tube, *b*, was submerged in the material contained in *D*; the air stream carried over and out of *C* would consequently be impregnated with the odor to be tested. By means of appropriate connections and the nozzle tube, *d*, three millimeters in diameter and bent in proper form, the air stream was led into the experiment chamber, *E*. Elastic suspension of the tube, *d*, automatically raised the tube

and allowed freedom of movement. For control tests there was provided a duplicate of the tube, *d*, which could be connected directly with *B*; thus avoiding any possible errors by contamination from the material in *D*. The chamber, *E*, was an open cylinder, the lower end resting in a shallow vessel containing soil easily changed and moistened. To eliminate other dis-

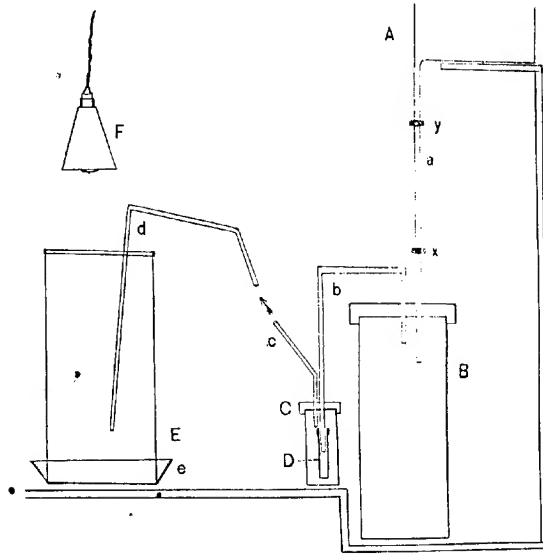


Fig. 1 A, reservoir; B, air chamber; C, odor chamber; D, vial; E, experiment chamber; F, lamp; a, water tube A to B; b, airtube B to C; c, outlet tube for odor stream; d, nozzle tube for chamber E; e, vessel containing sand; x, y, clamps.

turbing factors the cylinder was covered with black cloth. The illumination of the chamber, *E*, was by an electric lamp of 8 c.p. This was of advantage in orientation, as the toads are positively phototropic (Pearse '10).

The apparatus was arranged near the water supply in a basement room from which daylight could be excluded. Several other chambers similar to *E* were provided in order to adjust

the toads to the new conditions. Toads not accustomed to the chamber were at first much disturbed, reacting to various other influences. Attempts to crawl out of the chamber, to bite the end of the tube, *d*, or to be restless within the chamber were some of the reactions noted. After adjustment normal reactions were then exhibited, such as feeding or burrowing into the soil. The apparatus was kept well ventilated between periods of experimentation as well as during the testing periods.

### *2. Rate of flow of odor-stream*

The rate of flow of the air stream was made as uniform as possible. If allowed to flow uninterruptedly the 3000 cubic centimeters of air in *B* were displaced by the water from *A* in from 80 to 90 minutes. At this rate the flow is approximately 35 cubic centimeters per minute. That such a stream is not sufficiently strong to be perceptible can be demonstrated by placing the nozzle tube near the moistened lips or the tongue. The flow of the stream could also be judged from the frequency of escaping bubbles when the end of the tube was immersed in water.

The tests as carried out followed a uniform plan consisting of, first, a control test with air, and secondly, a trial proper with the odor stream.

### *3. Methods*

In the control test the toads were first subjected to the air stream coming from *B*. The nozzle tube, *d*, was directed toward various body regions, namely, flank, axillary region, anal region, and surface of the eye (as near as possible without contact). After this test for possible stimulation of integumentary sense organs, the air stream was next directed to the nostril to ascertain if there was any stimulation of the nasal epithelium. The test with the odor stream followed next and the same parts of the body were tested as before.

The toads used in the feeding experiments were used for the tests with the odor stream and were normal in all their activities.

The one exception was Toad No. 4, which seemed to be in a semi-hibernating condition during the entire period. Two other toads taken in the fall of 1912 were likewise tested with this apparatus. Although no two individuals were identical, the results of the trials agree in all essentials.

#### *4. Substances used in the tests*

Oil of cloves, oil of pennyroyal, oil of rose geranium, cedar oil, bergamot oil, aniline oil, carbolic acid, olive oil, castor oil, and cod liver oil were used, though some of the substances gave entirely negative results.

Trials were also made with the odor stream from food materials used by the toad. For these tests mealworm larvae, earthworms, dungworms, and cockroaches and other insects were put directly into the chamber, C and the air stream was allowed to carry over any odors that were present. In no case was there evidence of olfactory stimulation from these bodies.

#### *5. Experiments*

The attempt of Graber ('85) to determine the olfactory reactions in some of the amphibians proved unsatisfactory. Aronsohn ('86) and Gourewitsch ('83) noted the effect of odors on the rate of respiration. The animals, while confined in covered beakers, were exposed to odors of turpentine and eau de cologne with disturbing results.

Early in my own tests it was seen that chloroform, ammonia, ether, turpentine, formol or alcohol when introduced into the chamber were disturbing. Even though the air stream with these substances was not near the nostril, reactions followed very quickly and with the proximity of the stream to the nose the disturbance was increased.

Ammonia, chloroform and turpentine when directed upon the anterior head region induced the toad to jump away from the tube to one side or even out of the chamber. The odor of turpentine resulted in a very characteristic attitude, the head



being bent down between the forelegs, the body raised from the ground and best described as humpbacked. Ether or alcohol did not produce reactions so decidedly vigorous, though the toads retreated quickly from the tube.

The respiratory movement was retarded by all the substances that stimulated the olfactory end-organ. Tests made with the odor stream when the act of inspiration was suppressed were devoid of any resultant motor activity.

After the earlier tests with ammonia, ether, etc., the essential oils were used and some were found to be more effective in calling forth characteristic reactions than others. Some were entirely negative in effect, as, for instance, castor oil, cod liver oil, and olive oil.

What reactions could be considered as directly called out by the stimulation of the olfactory organ?

When the odor stream from oil of cloves or pennyroyal was allowed to spread over the anterior region of the head and presumably enter into the nasal cavity with the inspired air, the first motor act consisted of a slight bending down of the head, away from the nozzle of the tube, and a cessation of the respiratory movements. If the tube were removed quickly, the toads soon resumed the normal position and the respiration movements went on again with little interruption. If the odor stream was allowed to flow continuously upon the nostrils, respiration was entirely suppressed for some time. Often the toad made motions with the forelegs resembling wiping. When very much stimulated by the stream the animals moved away from the tube. The bending of the head was most noticeable when the toads were partly hidden in the sand. At such times respiration ceased, the head was bent more anteriorly and the animals endeavored to burrow down into the soil. The act of burrowing could be hastened by directing the tube repeatedly at the nostrils during such a test. This position of being partly buried in the soil was most favorable for observation. When thus buried the animals did not move about and the stream could be directed more accurately against the nostrils than at other times when the animals were free to move about.

The tests were carried out in periods of approximately five-minute durations, followed by intervals of rest varying in length of time. It sometimes happened that the animals were too restless to allow a fair interpretation of their reactions; in such cases the tests were discontinued. Other individuals again, were amenable to the trials for several hours without showing themselves to be disturbed. Precaution was taken that no odors entered the chamber otherwise than by means of the appropriate tube.

Records of the trials indicated what reaction followed the stimulus applied to the nostrils. The records shown in table 1 will serve as samples.

TABLE 1  
*Toad No. 1*

TIME	STIMULUS	REACTION
3.00	air	moved to one side
3.05	repeated	burrowing normally
	toad was allowed to rest fifteen minutes	
3.20	odor of oil of clove	head bent downward
	repeated	head bent downward
	repeated	wiped with right foot
	repeated	moved away to left
	repeated immediately	moved away to left
	repeated	wiped; attempted to climb
3.25	repeated	wiped again

*Toad No. 2*

10.30	air	at rest
	repeated	at rest
	repeated	at rest
10.35	repeated	at rest
	Toad allowed to rest five minutes	
10.40	odor of oil of pennyroyal	wiped
	repeated	head bent downward
	repeated immediately	head bent downward
	repeated	moved toward right
	repeated	head bent downward
	repeated immediately	head bent downward
10.45	repeated	head bent downward

Bergamot and cedar oil resulted in fewer reactions than oil of clove and oil of pennyroyal, showing them to be less stimulating than the other oils.

As already indicated, the stimulus was effective only while inspiration was in progress, for pressure of the stream was so slight that it could not force the odorous particles into the nostrils. The reactions were not characteristic of the different odors. It might be said that the reactions differed in degree, only, since there were only differences of vigor with which the reactions were executed. Even individuals differed in this respect at different times during the trial periods. Other factors such as temperature or tendency to hibernate may have had some influence in this aspect of the reactions. Absolute parity of the tests could of course not be obtained.

The relative effectiveness of the odors in stimulating the receptors was as follows: Most effective and approximately equal were oil of cloves, and oil of pennyroyal; less effective were oil of rose geranium and cedar oil; and least effective, with reactions infrequent, were bergamot oil and carbolic acid.

Odors from castor oil, olive oil, cod liver oil, living mealworms, earthworms, dungworms, cockroaches, decaying meat and decaying leaves in soil were without effect.

#### *6. Controls and operations*

The controls used in the tests seemed to be confirmatory of the presence of the olfactory function; yet a possibility existed that other receptors had been stimulated at the same time. The ophthalmic branch of the trigeminal nerve supplies the region of the head anterior of the nostrils, with some fibers possibly present within the nasal capsule. This being so, stimuli coming to this region of the head could call forth reactions easily through this branch as well as through the olfactory organ. To ascertain whether this branch had been stimulated several sets of experiments were tried.

The nostrils were closed by suturing with silk thread. Closing the nostril in this manner was of more serious consequence to the

toads than to fishes. This established an effective hindrance to the respiratory current, therefore it was found impracticable. If the stitches were not placed deeply, the muscular movements of the nostril soon caused the thread to cut through the margins. Furthermore, complete closure of the nostrils was apparently fatal in several preliminary trials.

Filling the nasal aperture with melted vaseline was only partly successful, entrance to the capsule being prevented by the nostril valves. The vaseline could be "troweled" into the opening. It was evident from the actions of the animals that the vaseline was discomforting. The toads showed decided restlessness and attempted to wipe their heads.

Some tests were made with toads so treated. When both nostrils had been successfully closed, no reactions traceable to odorous substances could be observed. If only one of the nostrils was closed, the odorstream, when directed upon the open nostril, was effective in causing a reaction.

Clove oil and pennyroyal were the substances used in the tests with the nostrils partly or wholly closed, these having been most efficient in calling forth reactions in the normal toads.

To be certain of the assumption that the reactions noticed with the odorstream were called forth by the stimulation of the olfactory receptor, operations of two kinds were performed on the toads.

In one of the operations, the olfactory tract was severed. In the other, it was necessary to section the ophthalmic branch of the fifth nerve. After etherization, the olfactory tracts were severed at the anterior border of the eyeball. The ophthalmic branch of the trigeminal was cut by piercing the integument of the optic capsule at the anterior inner angle of the orbit and cutting across the floor of the orbit. After recovery, only the specimens reacting normally to other stimuli were used for the tests.

Toads in which the olfactory tracts had been severed did not react to the odorstream. If partly buried in the sand, they were undisturbed by odors coming through the tube. The head was not drawn down, nor did they move away from the tube. One

peculiar action was noted in the toads so tested; when the stream was first directed to the nostrils, they sometimes would gasp, often repeatedly, but without interrupting the respiratory act.

The reactions of the toads in which the olfactory tracts had been severed are well exemplified in the record of Toad No. 1 (table 2).

The reactions of toads in which the ophthalmic branch of the trigeminal nerve has been cut is shown in table 3.

Since oil of cloves and oil of pennyroyal had given the best reactions in the normal toad these substances were used after the operations. As may be seen from the record above, the operation on the ophthalmic branch of the fifth nerve did not disturb the olfactory function.

Ether and chloroform did not affect the operated toads in any manner different from the normal animals.

After cutting the olfactory tract the animals were apparently less active than toads with the ophthalmic branch severed.

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TABLE 2  
Toad No. 1

TIME	STIMULUS	REACTION
1.10	odor of oil of clove	resting
	repeated often	moved forward
	repeated often	wiped once
1.15	with continuous odorstream	respiration normal
Toad	allowed to rest for ten minutes and	chamber well ventilated
1.25	odor of oil of clove	gapped at first
1.30	continued for five minutes	wiped once
	Allowed to rest ten minutes	
1.40	odor of oil of clove	
	continuous for four minutes	wiped once
1.45	further stimulation	gapped once
	Allowed to rest ten minutes	
1.55	odor of oil of clove	quiet; respiration suspended
	continuous odorstream	respiration resumed
2.00	continuous odorstream	no reactions
	Allowed to rest ten minutes	
2.10	odor of oil of clove	
2.15	continuous stream	changed position once and respiration normal

The latter were more responsive to the optic stimulations. The toads with the olfactory tract cut preferred to lie quietly in the sand. The feeding reactions were not inhibited by, either operation.

Some toads collected in the late summer of 1912, tested similarly, gave evidence coinciding with the data of the previous tests.

The results of these experiments show that odors stimulate the olfactory receptors, whereby certain motor activities of unquestionable value to the animals are called forth.

TABLE 3

TIME	STIMULUS	REACTION
9.35	odor of oil of clove	head down
	repeated	wiped with right forefoot and moved backward
	repeated	moved away from tube
	repeated	head down and moved backward
9.40	repeated	moved away from tube
	Toad allowed to rest five minutes	
9.45	odor of oil of clove	head down
	repeated	withdrew from tube
	repeated	head down
	repeated	wiped head twice
	Allowed to rest five minutes	
9.55	odor of oil of clove	head down, drew back
	repeated	head down
	repeated	head down
	repeated; applied to left nostril	wiped left side of head
10.00	repeated; applied to right nostril	wiped right side of head
	Allowed to rest twenty minutes	
10.20	odor of oil of clove	head down
	repeated	head down
	repeated	drew back
	repeated	head down
10.25	repeated	head down
	Toad allowed to rest five minutes	
10.30	odor of oil of clove	drew back, drew back
	repeated	jumped away
	repeated	drew back, drew back
	repeated	head down, head down
10.35	repeated	jumped away, drew back

## EXPERIMENTS WITH TADPOLES

*1. General*

Knauer ('75) emphasizes the fact that some of the larval anurans are less phytophagous than is commonly supposed and instances the fondness of toad tadpoles for animal food. Holmes ('07) also states that decomposing insects, earthworms, etc., are acceptable as food to frog tadpoles. That certain European anurans feed indiscriminately like the earthworm is a well-known fact. Whether vegetable material must not be in the early stages of decomposition before becoming available as food for tadpoles has not yet been determined. It is certain that many unicellular and filamentous algae may be ingested repeatedly before digestion is complete.

Nagel ('94) denied the olfactory function to aquatic vertebrates. His theoretical objections have been refuted for the fishes by the work of Parker ('10; '11), Sheldon ('11), and Cope-land ('12). The question still remains open as regards amphibians and its solution depends upon suitable material and methods. This was reason sufficient to attempt an investigation of the olfactory function in the tadpole of the toad. Preliminary trials gave evidence at once that the tadpoles were responsive to the presence of decaying animal matter in their immediate vicinity. Tadpoles placed in a vessel containing filtered water for a day or two, the feces being removed frequently, soon come to be in a state of hunger. Water not filtered contained all times organic material enough to form a delicate film upon the walls of the vessel. This film of partly decomposing organic matter is a source of food for the tadpoles and must be removed. For like reasons the feces must be taken away.

In the preliminary trials particles of dead earthworms, dead fish or bits of meat undergoing decomposition were placed in the vessel containing the hungry tadpoles. Such particles of food were quickly found by the tadpoles.

The feeding trials were performed on tadpoles of three successive years. The first set was obtained at Woods Hole, Massachusetts, on August 10, when the tadpoles were about to

metamorphose. Some of the larvae had one, others both pairs of appendages well developed, and a few specimens passed through the final phases after having been brought into the laboratory. They were kept in shallow vessels with some of the bottom detritus from their original habitat. Experimentation and natural causes gradually diminished the numbers available for the trials, but the tadpoles appeared to be normal in their activities at all times. The tadpoles surviving through the period of experimentation were killed by accident in the month of January following.

The second set were tadpoles taken in May of the following year in the vicinity of Cambridge. They did not lend themselves well to experimentation. Conditions appeared to be unfavorable for them in the laboratory. Experiments therefore were carried on with but small numbers, but so far as these went they were corroborative of those from the first set.

The tadpoles included in the third set were taken in June, 1913, from two different localities: namely, Cambridge and Woods Hole, Massachusetts. The tadpoles were experimented with particularly to show that the olfactory reactions might be completely checked by a certain procedure and then revived.

## 2. Methods

The method of procedure in the trials was as follows: The tadpoles were placed in filtered water for twenty-four to forty-eight hours before experimentation began. Then the food was introduced into the vessel; the reactions being noted in accordance with the shifting of the food placed in the vessel.

Food used in the tests consisted of particles of fish, earthworm, or meat in decomposition. At first the food without any envelope was placed free in the vessel, but later it was wrapped in cloth. In other trials again, two packets were placed in the water; one containing the food, the other without it.

Very little difficulty was experienced in noting the reactions of the tadpoles. When the two packets were presented, the tadpoles distinguished quickly the one containing the food.



When confined to shallow vessels, the actions of the tadpoles of the toad and of the frog of approximately the same stage, were somewhat different. In general, the frog tadpoles swim along the vessel walls. The toad tadpoles, on the other hand, move in all directions over the bottom or near the upper surface of the water.

When toad tadpoles came into the vicinity of the food, either covered or open, the reactions seemed to indicate stimulation of some kind. They swam from side to side, and often when near the food would turn directly to it. When the food was found the tadpoles attacked it very eagerly. When two packets had been introduced the tadpoles did not remain upon the packet without food nor did they nibble it as they did the one containing food.

### 3. Experiments

*a. First set of tadpoles.* The records of several of the trials are here given in detail:

#### TIME

- 8.20 Food packet placed in shallow dish containing eight tadpoles
- 8.22 Food found by first tadpole
- 8.23 Food found by second tadpole
- 8.39 Food found by six tadpoles
- 8.50 The position of the food was changed
- 8.55 The food found by three tadpoles
- 8.57 The food found by five tadpoles
- 8.05 The food found by seven tadpoles
- In another trial a small piece of fish not covered was placed in the dish.
- 10.30 Food placed in dish containing eight tadpoles
- 10.40 Food found by six tadpoles
- In another trial two packets were placed in the dish, one with food, the other without.
- 2.35 Food placed in dish with six tadpoles
- 2.43 All the tadpoles had found the food and were nibbling
- None of the tadpoles were at the false packet.
- 2.55 Reversed position of the two packets
- 3.20 Food found by five tadpoles
- In a similar trial with two packets, one containing fresh meat, the other without.
- 10.42 Packets placed in dish with fifteen tadpoles
- 10.50 Food found by twelve tadpoles
- 10.56 Reversed position of packets
- 11.05 Food packet found by ten tadpoles

The totals of the trials carried on with the larva of the first set gave these results: In 160 trials, food packet was found 120 times. Trials carried on from time to time up to the accidental loss of the tadpoles resulted in similar ratios.

*b. Second set of tadpoles.* The conditions were unfavorable for keeping these tadpoles and because of this circumstance only a small number of individuals were available. The trials were carried out in a manner similar to those for the first set.

It needs only be said that the results were similar to those obtained from the experiments of the first set. Precautions were taken with this set to have the two packets identical in appearance, and to transpose them in position. To avoid the accidental finding of the packets as much as possible, they were placed some distance from the sides of the vessel. The toad tadpoles swam more rapidly than the frog tadpoles, and were also more erratic in their movement in the water. It could be easily determined whether they were influenced by the presence of the food mass near them. In a few cases only did there seem to be a visual stimulus influencing the tadpoles to react; such being occasioned by the very lively actions of other tadpoles already at work on the food mass.

*c. Third set of tadpoles.* For the purpose of verifying the results of the previous experiments, similar experiments were performed with the tadpoles of the third set in June and July of the third season. As stated, these tadpoles were obtained from two different localities and kept successfully in the laboratory, metamorphosis being deferred for the time being. The tadpoles were unquestionably those of *Bufo americanus*, having been obtained earlier than the time when the mating call of Fowler's toad was heard.

The food used in these experiments was dead and partly decomposed frog tadpoles, earthworms, and beef liver. These materials were either placed freely in the vessel or covered with cheesecloth one or two layers in thickness. In a similar way, as previously described, two packets were used in some of the trials; one containing the food, the other being identical in appearance but without food.

A number of trials in which the food was placed in the vessel with the tadpoles showed that the reactions of the tadpoles depended somewhat on the size of the vessel. In a small vessel the time necessary for the food to be found by a certain number of the tadpoles was usually less than in a larger vessel. The same is true if the food is placed near the side of the vessel, say within an inch of the side wall; in such a case the animal swimming through the zone impregnated by the odor is influenced more quickly than when the food is centrally placed.

In the trials with two packets discrimination was very evident in the actions of the tadpoles. In the beginning of the trial the reactions were at all times apparently without choice. After the packets had been allowed to remain in the water for a short time, the tadpoles always endeavored to feed from the packet containing the food. Even if the tadpoles rested on the 'dummy' packet very little attempt was made to nibble. Transposition of the two packets was accompanied by a corresponding redistribution of the tadpoles.

This last method was modified several times, by exchanging the envelopes of the packets, or by substituting an envelope saturated with the odor of the food material for the food itself. When this was done, the tadpoles congregated upon the food-saturated envelope, finding it as they did the food open in the water, or when contained in the envelope and allowed to remain for some time.

An attempt was made to determine whether the tadpoles would orient themselves to an odor-saturated waterstream which was allowed to flow into a vessel containing them; but this experiment was fruitless of results, although there was some slight evidence that the current was especially stimulating.

Corroborative of the earlier findings of the experiments on the two previous sets, experiments carried out with the third set were of greater value in this study in what may be called operative tests.

To determine whether the reactions of the toad tadpoles as already described are due solely to the stimulus received

by the olfactory organ, attempts were made to inhibit such stimulation in various ways. This was found to be rather difficult and in the light of the earlier attempts the evidence was not very convincing. The diminutive size of the tadpoles is the principal factor militating against successful operations.

Cutting the olfactory tract is the method by which inhibition may be made absolutely certain. In this method the chief difficulty lies in determining the proper degree of anesthetization for the operation and subsequent revival of the animal. Chloroform of 0.1 per cent was used for this purpose, as the tadpoles could be brought into a vessel with fresh water and revived. After being anesthetized the tadpoles were bedded in a bit of absorbent cotton held in the hand, and with a needle the cranial case was pierced in the median line anterior to eyes. The shock attendant upon the operation or the manipulation was disastrous in most cases, and only a few specimens survived. Within a few days these tadpoles became less vigorous, and finally all succumbed.

The few individuals so operated upon and tested for reactions resulting from stimulation to the olfactory sense organ did not give sufficient evidence from which satisfactory conclusions could be drawn.

In another manner the inhibition of the stimulation and the reactions was also attempted. The external nares of the toad tadpole are comparatively large and it was possible to fill the nares with white vaseline, the tadpoles being bedded in moist absorbent cotton. Tadpoles so treated showed the presence of the vaseline to be disturbing in effect. The plugs prevent the respiratory stream from entering the nasal openings, the swimming movements are not so vigorous as under normal conditions, the chief endeavor of the tadpole being to free itself from the disturbing material. The temperature of the water is to be taken into account here, as the vaseline could be removed quite readily by placing the tadpoles in water slightly warmed. As soon as the vaseline has been dissolved the tadpoles again act in normal manner. This method of treatment was made use of in the first

and second sets, but on account of the comparatively small numbers at hand, the results could not be considered as conclusive.

With the material of the third set the chief aim was to determine whether the presence of the vaseline plugs was beyond doubt inhibitive of the apparent olfactory reactions. Some operations of cutting the tract were carried out, but the after effects were in most cases disastrous to the tadpoles, and therefore special stress was placed on plugging the nasal openings.

Making use of the same individual tadpoles in the successive trials, these groups were isolated in filtered water for some time previous to each trial, and tested repeatedly for reactions to the food as presented (without an envelope) while the nasal openings were in normal condition, or when filled with the vaseline plug.

The trials with the tadpoles in this series were carried out in the following manner: The tadpoles were isolated in filtered water for at least twenty-four hours, in a few cases forty-eight hours, but not beyond this, as the tadpoles showed that a longer period without food was disastrous to them. After isolation for the designated length of time, the tadpoles were tested for the presence of food in the water; immediately after this the nasal plugs were put in, and the tadpoles transferred to a vessel free of food or vaseline. The food was then introduced. Unless the food mass was placed immediately in the proximity to the tadpoles they exhibited no such activities as they previously had. When swimming and moving about in the vessel, there were no such positive attempts to find the food as when normal. The trials were repeated several times with different groups of individuals, in each case allowing several days to intervene between the trials, the tadpoles being provided with food and water from the stock.

The tadpoles showed no bad effects from the plugs of vaseline in the nasal openings, reacting freely in the later trials as well as in the earlier experiments.

Tests made with the tadpoles having the plugs in the nares showed them unable to discriminate between two packets one of which contained food, while previous to the plugging definite choice had been made by them in favor of the food packet.

The conclusion reached from these experiments as described for the different broods of tadpoles is that the presence of the nasal plug is effective in inhibiting any stimulus coming to the sense organ and consequently no corresponding reaction follows.

#### DISCUSSION

##### *1. Toads*

In its method of obtaining food the toad seems to respond to the visual stimulus entirely. This stimulus is apparently effective only when it involves motion. It is not always followed by perfect reaction, for substances inappropriate as food are often taken accidentally. Rejection of such material occurs in compliance with mechanical or tactile stimulation.

Nor does the gustatory function appear to be of any importance in feeding. Gaupp ('04) does not consider the function of the epithelial endplates of the mouth cavity to be established. He refers to Bethe as bringing forward the best evidence favoring them as tactile organs. It has been shown that strong solutions of picric or acetic acid applied to the epithelium of the mouth cavity will cause appropriate motor activity. Such reactions take place without reference to the point of application of the acid, whether this be in regions supplied with endplates or devoid of them. The latent period between stimulus and reaction is of appreciable duration.

These considerations speak against the belief that these organs are gustatory in function.

Until the contrary is proven, the similarity of food materials of frogs and toads argue for similarity in structure of the epithelium of the mouth. Observations and feeding experiments tend to confirm this view. Food does not remain in the mouth cavity any great length of time. Deglutition follows almost instantly, and therefore the sense of taste would be of minor importance. Under certain conditions regurgitation may occur. If substances disagreeable to taste are taken into the mouth the animals might be expected to resort to this expediency more often.

In no case in the course of the feeding experiments was cognizance taken of the unusual substances coming into the mouth to the degree that food was ejected or regurgitated. The experience cited by Knauer ('75) of toads refusing decomposing earthworms is probably referable to tactile stimulation. The nature of the organisms serving as food for the toads under natural conditions is such, that materials differing in texture markedly from the normal might be sufficient to cause refusal.

To establish any connection between food used by toads and the possible odors inherent to the food seems difficult at present. Although evidence is negative, this is qualified by the fact that the data are really not sufficient to establish any conclusion on this question. Natural foods are apparently taken indiscriminately. Stimulation of the receptors may take place; the presence of such stimulation and the effect are not yet demonstrable.

Our inability to recognize the quality of particular motor reactions following certain stimulations does not argue against the absence or refinement of reactions.

The experiments of Graber ('85) were such as to allow no great value to be attached to them. The unmodified methods used for forms differing so greatly in phylogenetic position and in habit with the very doubtful reactions as recorded, speak against the acceptance of his data as important.

To a similar degree the experiments of Aronsohn ('86) on odors and respiration are of little value in indicating the use of the olfactory organ in anurans. His experiment does not preclude stimulation of the trigeminal nerve. His choice of substances and the manner of experimentation favor the possibility that the fifth nerve is involved. The instance mentioned by Conradi ('01) has not had confirmation of any kind. In the experiments described, the presence of abnormal odorbearing substances has not given origin to stimulation sufficiently strong to inhibit the desire for food.

In all experiments carried out with the lower animals and their reactions toward solutions or vapors there exists the possibility that solutions or vapors are more dilute than was in-

tended. Whether stimulation is to take place in air or water, it is extremely difficult to work with solutions or gases absolutely standardized. The results of the experiments as conducted show that odors when in relation with food are not sufficiently deterrent in action to compel the toad to refuse such food. Odors of natural surroundings may stimulate the toads to certain reactions. At present there is no evidence that odors of soil or water are effective in any degree on the olfactory organ of the toad.

## 2. Tadpoles

In contrast with the tadpoles of the frog, the toad tadpole may be claimed to possess an olfactory sense, and possibly to a much greater degree than might be supposed. Anatomically considered the two species appear similar. Differences make themselves evident in a closer study of certain parts. The nasal openings in the toad tadpole are relatively larger than in the frog tadpole. The water stream into the nasal passage of the toad tadpole is therefore of greater magnitude than in the frog tadpole.

Contrary to Exner ('78), as quoted by Gaupp ('04), the nasal openings in both species serve for the incurrent water stream. If to this stream are added other substances: dilute solutions of methylene blue or particles of carmine, the toad tadpoles are very quick to respond to the stimulus. Such substances added to the water current flowing into the nasal chamber of the tadpoles of the frog (*Rana virescens* and *R. catesbiana*) produce similar reactions. Toad tadpoles react at the immediate entrance of the first substances into the nostril; tadpoles of the frog will permit the stream to flow into the nose for a long period, reacting much more slowly to the stimulus. Probably there is a mechanical stimulus from the carmine which sets free the response.

It is more than probable that toad tadpoles recognize certain foods and their odors. When given the choice as between decaying animal matter and decaying plant substances the former is preferred.



In the series of tests there was always positive reaction in the presence of the animal food when opportunity for such choice was given. Organs of taste have not been demonstrated in the mouth of the tadpole.

On the other hand, the differentiation of the nasal epithelium into the characteristic olfactory organs and the supporting structures takes place very early and we may safely assume the reactions noted to be the result of stimulation of the olfactory receptors.

### *3. Conclusion*

The anurans have been called microsmatic animals; such distinction being based on histological and anatomical comparison with the animals classed as macrosmatic.

The nasal organ of the Anura is a common respiratory and olfactory organ, and in this respect conforms to the organ possessed by the higher vertebrates.

A chambered nasal cavity of considerable magnitude supplied extensively with olfactory epithelium and adequate connections with the central nervous system, predicate functional activity of the sense organ.

The life habits and the phylogenetic position of the anurans suggest that such functional activity not only be present but adequately developed. The presence of receptor organs identical in structure with those found in the higher vertebrates further postulates that functions peculiar to these structures be identical.

The receptor peculiar to the olfactory organ of all vertebrates consists of a neurone whose cell body is peripheral in position. The distal portion of the neurone is characterized by the protoplasmic processes projecting above the level of the surrounding cells, while the proximal end is attenuated and gives rise to one of the fibers of the olfactory nerve. This type of receptor is directly comparable structurally with neurone cells found in the epidermis of many invertebrates (Parker '12). Neurones such as these, found in some of the invertebrates are distinct portions of the receptor-effector system and have been demonstrated as extremely sensitive to chemical stimuli. That

such-receptors, olfactory in function and responding only to stimuli when the cell surfaces are dry were peculiar only to air breathing animals was the earlier assumption.

The work of Aronsohn ('86) and Veress ('03) has shown that in man and the higher vertebrates the olfactory epithelium is bathed by glandular secretions and whatever stimulus reaches the receptor must do so in the form of solutions.

More recently Baglioni ('09), Parker ('10; '11), Sheldon ('11) and Copeland ('12) have demonstrated in aquatic animals the stimuli inducing certain reactions to be identical with those noted in the air-breathing animals.

If this assumption is valid, as it appears to be, that the olfactory receptor is the simplest and least differentiated of receptor neurones and stimulated by extremely dilute solutions, we should find the olfactory organ in Anura readily stimulated.

That well defined and characteristic motor reactions have not yet been recognized as results due to stimuli varying in quality may be due to lack of observational data and methods of experiment.

#### SUMMARY

1. There is no evidence that toads react to olfactory stimuli pertaining to soil, water, etc.
2. The character of the food is not differentiated by attendant odors to the degree that the adult toad thus distinguishes it.
3. Substances of unusual character and odors, when associated with food, do not stimulate the olfactory organs in such a manner as to bring the toad to refuse the food.
4. The presence of such substances in close proximity to the toad, and invisible because of darkness are not repellent in effect on the toad.
5. Odorstreams specific in character, made to flow over and into the nasal openings stimulate the olfactory sense-organ; such stimulation causing definite motor activities to follow.
6. Appropriate operations are confirmatory that the stimulation by such odorstream is olfactory. Section of the olfactory tract inhibits the reactions. Olfactory stimulation and reactions are

not affected by section of the ophthalmic branch of the trigeminal nerve.

7. Under circumstances allowing discrimination, the tadpoles of the toad prefer animal foods.

8. Such discrimination appears to rest upon the appropriate stimulation of the olfactory receptor.

9. Tadpoles of the toad show by proper reactions that animal food is recognized, although not visually perceptible.

10. The receptor organ so stimulated must be a distance receptor and thus is olfactory in function.

11. In the metamorphosed toad the visual stimulus is the principal and guiding factor in procuring food. Therefore, it is inhibitory in relation to other stimuli and their resultant reactions.

POSTSCRIPT. Since the preparation of this paper, Copeland has published in *The Journal of Animal Behavior*, vol. 3, pp. 260 to 273, an account of the olfactory reactions of the newt *Diemyscylus* and has shown that this amphibian can scent food under water as a fish does.

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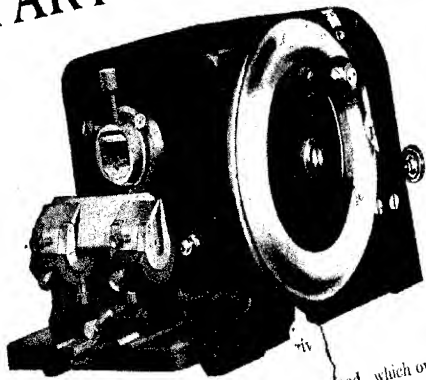
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# SUBJECT AND AUTHOR INDEX

- A** LLEE, W. C. Certain relations between rheotaxis and resistance to potassium cyanide in *Isopoda*. . . . . 397
- Amphibian embryos. Experimental evidence concerning the determination of posture of the membranous labyrinth in. . . . . 149
- Amphibian larvae to light. The reactions of normal and eyeless. . . . . 195
- Amphibians. Olfactory reactions in. . . . . 617
- Anesthetics. Antagonism between salts and IV. Inactivation of hypertonic sea-water by anesthetics. . . . . 591
- Antagonism between salts and anesthetics. IV. Inactivation of hypertonic sea-water by anesthetics. . . . . 591
- Arbacia. Studies of fertilization. VI. The mechanism of fertilization in. . . . . 523
- Asplanchna amphora*. Transmission through the resting egg of experimentally induced characters in. . . . . 347
- B** AITSELL, GEORGE ALFRED. Experiments on the reproduction of the hypotrichous Infusoria. II. A study of the so-called life cycle in *Oxytricha fallax* and *Pleurotricha lanceolata*. . . . . 211
- Bee. The olfactory sense of the honey. . . . . 265
- Birds. A further study of size inheritance in ducks, with observations on the sex ratio of hybrid. . . . . 131
- BORING, ALICE M. and PEARL, RAYMOND. The odd chromosome in the spermatogenesis of the domestic chicken. . . . . 53
- Bursaria to food. The relation of I. Selection in feeding and in extrusion. . . . . 1
- C** H A R A C T E R S in *Asplanchna amphora*. Transmission through the resting egg of experimentally induced. . . . . 347
- Chicken. The odd chromosome in the spermatogenesis of the domestic. . . . . 53
- CHILD, C. M. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. VII. The stimulation of pieces by section in *Planaria dorotocephala*. . . . . 413
- Chromatin in hybrids between *Fundulus* and *Ctenolabrus*. The behavior of the. . . . . 501
- Chromosome in the spermatogenesis of the domestic chicken. The odd. . . . . 53
- Conjugating and nonconjugating races of *Paramecium*. On so-called. . . . . 337
- Ctenolabrus*. The behavior of the chromatin in hybrids between *Fundulus* and. . . . . 501
- Currents produced by sponges. On the strength and the volume of the water. . . . . 443
- Cyanide in *Isopoda*. Certain relations between rheotaxis and resistance to potassium. . . . . 397
- D** U C K S, with observations on the sex ratio of hybrid birds. A further study of size inheritance in. . . . . 131
- E** G G of experimentally induced characters in *Asplanchna amphora*. Transmission through the resting. . . . . 347
- Embryos. Experimental evidence concerning the determination of posture of the membranous labyrinth in amphibian. . . . . 149
- Extrusion. The relations of Bursaria to food. I. Selection in feeding and in. . . . . 1
- F** E E D I N G and in extrusion. The relations of Bursaria to food. I. Selection in. . . . . 1
- Fertilization of *Nereis*. The effect of radium radiations on the. . . . . 85
- Fertilization. Studies of VI. The mechanism of fertilization in *Arbacia*. . . . . 523
- Fundulus* and *Ctenolabrus*. The behavior of the chromatin in hybrids between. . . . . 501
- H** E M A L nodes. The supposed experimental production of hemolymph nodes and accessory spleens. V. Studies on. . . . . 241
- Hemolymph nodes and accessory spleens. The supposed experimental production of V. Studies on. . . . . 241
- Honey bee. The olfactory sense of the. . . . . 265
- Hybrid birds. A further study of size inheritance in. . . . . 131
- Hybrids between *Fundulus* and *Ctenolabrus*. The behavior of the chromatin in. . . . . 501
- Hybrids. Modes of inheritance in teleost. . . . . 447
- Hypotrichous Infusoria. Experiments on the reproduction of the II. A study of the so-called life cycle in *Oxytricha fallax* and *Pleurotricha lanceolata*. . . . . 211
- I** N F U S O R I A. Experiments on the reproduction of the hypotrichous II. A study of the so-called life cycle in *Oxytricha fallax* and *Pleurotricha lanceolata*. . . . . 211
- Inheritance in ducks, with observation on the sex ratio of hybrid birds. A further study of size. . . . . 131
- Inheritance in experimental reproduction. Studies on the dynamics of morphogenesis and VII. The stimulation of pieces by section in *Planaria dorotocephala*. . . . . 413
- Inheritance in teleost hybrids. Modes of. . . . . 447
- Inheritance. Multiple factors in *Mendelini*. . . . . 177
- Isopoda*. Certain relations between rheotaxis and resistance to potassium cyanide in. . . . . 397
- L** A B Y R I N T H in amphibian embryos. Experimental evidence concerning the determination of posture of the membranous. . . . . 149
- Larvae to light. The reactions of normal and eyeless amphibian. . . . . 195
- LATOURN, HENRY. The reactions of normal and eyeless amphibian larvae to light. . . . . 195
- Light. The reactions of normal and eyeless amphibian larvae to. . . . . 195
- LELLIE, FRANK R. Studies of fertilization in *Arbacia*. VI. The mechanism of fertilization in. . . . . 523
- LELLIE, RALPH S. Antagonism between salts and anesthetics. IV. Inactivation of hypertonic sea-water by anesthetics. . . . . 591
- LUSD, E. J. The relations of Bursaria to food. I. Selection in feeding and in extrusion. . . . . 1

- MacDOWELL, E. C.** Multiple factors in Mendelian inheritance..... 177
- McInDoo, N. E.** The olfactory sense of the honey bee..... 265
- Membranous labyrinth in amphibian embryos.** Experimental evidence concerning the determination of posture of the..... 149
- Mendelian inheritance.** Multiple factors in..... 177
- MEYER, ARTHUR WILLIAM.** The supposed experimental production of hemolymph nodes and accessory spleens. V. Studies on hemal nodes..... 241
- MITCHELL, CLAUDE W., and POWERS, J. H.** Transmission through the resting egg of experimentally induced characters in *Asplanchna amphora*..... 347
- Morphogenesis and inheritance in experimental reproduction.** Studies on the dynamics. VII. The stimulation of pieces by section in *Planaria dorotocephala*..... 413
- MORRIS, MARJARET.** The behavior of the chromatids in hybrids between *Fundulus* and *Ctenolabrus*..... 501
- NEREIS.** The effect of radium radiations on the fertilization of..... 85
- NEWMAN, H. H.** Modes of inheritance in teleost hybrids..... 447
- Nodes and accessory spleens.** The supposed experimental production of hemolymph V. Studies on hemal nodes..... 241
- Nodes.** The supposed experimental production of hemolymph nodes and accessory spleens. V. Studies on hemal nodes..... 241
- OLFACTORY reactions in animals..... 617**
- Olfactory sense of the honey bee.**..... 265
- Oxyticha fallax and Pleurotricha lanceolata.** Experiments on the reproduction of the hypotrichous Infusoria. II. A study of the so-called life cycle in..... 211
- PACKARD, CHARLES.** The effect of radium radiations on the fertilization of *Nereis*..... 85
- Paramaecium.** On so-called conjugating and non-conjugating races of..... 237
- PARKER, G. H.** On the strength and the volume of the water currents produced by sponges..... 443
- PEARL, RAYMOND, BOHNS, ALICE M., and** The odd chromosome in the spermatogenesis of the domestic chicken..... 53
- PHILLIPS, JOHN C.** A further study of size inheritance in ducks, with observations on the sex ratio of hybrid birds..... 131
- Planaria dorotocephala.** Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. VII. The stimulation of pieces by section in..... 413
- Pleurotricha lanceolata.** Experiments on the reproduction of the hypotrichous Infusoria. II. A study of the so-called life cycle in..... 211
- Potassium cyanide in Isopoda.** Certain relations between rheotaxis and resistance to..... 397
- POWERS, J. H., MITCHELL, CLAUDE W., and** Transmission through the resting egg of experimentally induced characters in *Asplanchna amphora*..... 347
- RADIATIONS on the fertilization of Nereis.** The effect of radium..... 85
- Radium radiations on the fertilization of Nereis.** The effect of..... 85
- Ratio of hybrid birds.** A further study of size inheritance in ducks, with observations on the sex..... 131
- Reactions in amphibians.** Olfactory..... 617
- Reactions of normal and eyeless amphibian larvae to light.** The..... 195
- Reproduction.** Studies on the dynamics of morphogenesis and inheritance in experimental VII. The stimulation of pieces by section in *Planaria dorotocephala*..... 413
- Resistance to potassium cyanide in Isopoda.** Certain relations between rheotaxis and resistance to potassium cyanide in Isopoda. Certain relations between..... 397
- RISER, JONATHAN.** Olfactory reactions in amphibians..... 617
- SALTS and anesthetics.** Antagonism between IV. Inactivation of hypertonic sea-water by anesthetics..... 591
- Sense of the honey bee.** The olfactory..... 265
- Sex ratio of hybrid birds.** A further study of sex inheritance in ducks, with observations on the..... 131
- Size inheritance in ducks, with observations on the sex ratio of hybrid birds.** A further study of..... 131
- Spermatogenesis of the domestic chicken.** The odd chromosome in the..... 53
- Spleens.** The supposed experimental production of hemolymph nodes and accessory V. Studies on hemal nodes..... 241
- Sponges.** On the strength and the volume of the water currents produced by..... 443
- STUEBNER, GEORGE L.** Experimental evidence concerning the determination of posture of the membranous labyrinth in amphibian embryos..... 149
- TELEOST hybrids.** Modes of inheritance in..... 447
- WOODRUFF, LORANDE LOSS.** On so-called conjugating and non-conjugating races of *Paramaecium*..... 237

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CONTENTS VOLUME 16

• • NO. 2 MARCH •

FRANKLIN PARADISE JOHNSON. The development of the rectum. Twenty-five figures.....	1
B. F. KINGSBURY. The interstitial cells of the mammalian ovary: <i>Felis domestica</i> . Sixteen figures.....	59
S. WALTER RANSON. The tract of Lissauer and the substantia gelatinosa <i>Rolandii</i> . Eleven figures.....	97

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